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University of Southern California	January 27, 1949
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Antibacterial Activity of d-Usnic Acid and Related Compounds on *M. tuberculosis.*

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Usnic acid, a lichen acid isolated over a century ago, has been the subject of active study at various schools of chemistry in respect to structure. That it is a promising antibiotic particularly antagonistic to *M. tuberculosis in vitro* and *in vivo* was discovered by Marshak and has since been confirmed *in vitro* by Stoll, Brack and Renz, Barry and more recently by Shibata and Ukita.[1-4]

The excellent antibacterial characteristics of usnic acid are somewhat offset by its poor water solubility which leads to difficulty in administration to animals and a considerable degree of toxicity. It is therefore of interest to ascertain whether or not these drawbacks could be counteracted by suitable operations on the molecular architecture of usnic acid. Accordingly, a series of derivatives and degradation products of d-usnic acid have been prepared in the hope that one or other of these compounds (No. 2-10, No. 15-18 and No. 21) might possess more advantageous pharmacological and antitubercular properties than usnic acid itself; these could conceivably arise in the animal organism as a result of the customary detoxification mechanisms. More specific attempts to prepare derivatives which

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1 Marshak, A., *U. S. Public Health Repts.*, 1947, **62**, 3.

2 Shibata, S., and Ukita, T., *Japanese Med. J.*, 1948, **1**, 152.

3 Stoll, A., Brack, A., and Renz, V., *Experientia*, 1947, **3**, 115.

4 Barry, V. C., *Nature*, 1947, **160**, 800.

TABLE I.

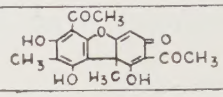
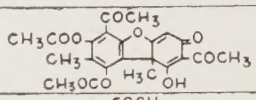
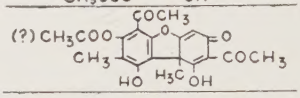
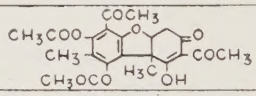
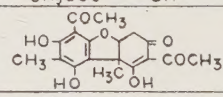
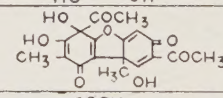
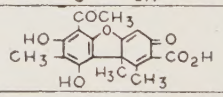
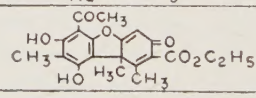
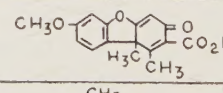
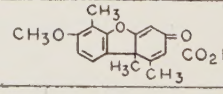
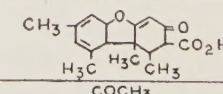
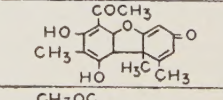
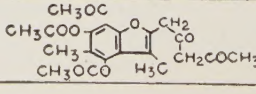
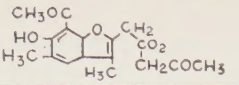
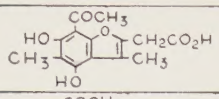
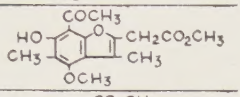
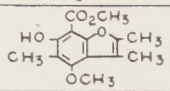
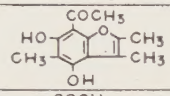
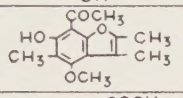
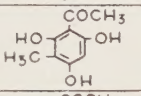
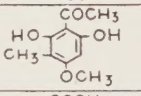
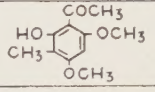
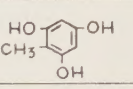
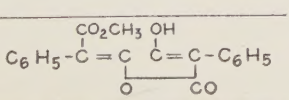
Serial No.	Substance	Structural formula where known in literature	Solubility	Growth of *M. tuberculosis* H 37 Rv — 0 means no growth — Days after inoculation	Drug conc. in μg/cc 20	10	2	0.5
0	(Control)			10	4	4	4	4
				15	4	4	4	4
				20	4	4	4	4
1	*d*-Usnic Acid		c	10	0(0)	0(2)	4(4)	4(4)
				15	0(0)	0(3)	4(4)	4(3)
				20	0(0)	0(3)	4(4)	4(3)
2	rac-Usnic Acid	,,	c	10	0	0	3	4
				15	0	0	4	4
				20	0	0	4	4
3	*d*-Diacetyl Usnic Acid		s	10	0	1	4	4
				15	0	2	4	4
				20	0	2	4	4
4‡	*d*-Monoacetyl Usnic Acid		p	10	0	0	3	4
				15	0	0	3	4
				20	0	0	3	4
5	*d*-Usnamide		p	10	2	3	4	4
				15	2	3	4	4
				20	3	3	4	4
6	*d*-N_5 Methylusnamide		p	10	4	4	4	4
				15	4	4	4	4
				20	4	4	4	4
7	*d*-Diacetyl Dihydro Usnic Acid		s	10	3	4	4	4
				15	3	4	4	4
				20	3	4	4	4
8‡	*l*-Dihydro Usnic Acid		s	10	0	1	4	4
				15	0	2	4	4
				20	0	3	4	4
9	*d*-Usnonic Acid		s	9	2	4	4	4
				12	2	3	4	4
				18	2	3	4	4
10‡	Usnolic Acid		s	10	3	4	4	4
				15	3	4	4	4
				20	4	4	4	4
11	Ethyl Usnolate		s	9	1	3	4	3
				12	2	2	4	4
				18	2	2	4	4
12†	6′-Methoxy-3′,3-dimethyl-2′,3′-dihydro-benzofurano (2′,3′,5,4-)-$\Delta^{2:5}$ cyclohexadienone-2-carboxylic Acid		s	9	2	3	3	3
				12	2	3	3	4
				18	2	3	3	3
13†	6′Methoxy-3′,7′,3-trimethyl-2′,3′-dihydro-benzofurano (2′,3′,5,4-)-$\Delta^{2:5}$ cyclohexadienone-2-carboxylic Acid		s	9	2	3	3	3
				12	2	3	3	4
				18	2	3	3	3
14†	3′,4′,6′,3-Tetramethyl-2′,3′-dihydro-benzofurano (2′,3′,5,4-)-$\Delta^{2:5}$ cyclohexadienone-2-carboxylic Acid		s	9	3	3	3	3
				12	3	3	3	3
				18	3	3	3	3
15	Decarbousnol		s	10	4	4	4	4
				15	4	4	4	4
				20	4	4	4	4
16	Diacetyl decarbousnic Acid		p	10	4	4	4	4
				15	4	4	4	4
				20	4	4	4	4

TABLE I (Continued)

17	Decarbousnic Acid		p	10 15 20	3 3 3	3 4 4	4 4 4	4 4
18	Usnetic Acid		s	9 12 18	4(3) 4(3) 4(3)	4(4) 4(3) 4(3)	4(4) 4(3) 4(3)	4(4) 4(4) 4(4)
19†	Methylusnetate-4-methyl ether		p	9 12 18	3 2 2	3 4 3	4 4 4	4 4 4
20†	Methyl 2,3,5-trimethyl-4-methoxy-6-hydroxy cumarone-7-carboxylate		p	9 12 18	3 3 3	3 4 4	4 4 4	4 4 4
21	Usnetol		c	9 12 18	4(1) 4(2) 4(4)	4(1) 4(3) 4(4)	4(4) 4(4) 4(4)	4(4) 4(4) 4(4)
22†	Usnetol-4-methyl ether		p	9 12 18	4 4 4	4 4 4	4 4 4	4 4 4
23†	Methyl acetophloroglucinol		s	9 12 18	4 4 4	4 4 4	4 4 4	4 4 4
24†	Methyl acetophloroglucinol-4-methyl ether		s	9 12 18	4 4 4	4 4 4	4 4 4	4 4 4
25†	Methyl acetophloroglucinol-4,6-dimethyl ether		p	9 12 18	4 4 4	4 4 4	4 4 4	4 4 4
26†	Methyl phloroglucinol		s	9 12 18	4 4 4	4 4 4	4 4 4	4 4 4
27	Diacetyl usnic acid ethoxylate		s	9 12 18	3 3 3	3 3 4	4 4 4	4 4 4
28	Sodium *d*-usnate		s	10(9) 15(12) 20(18)	0(0) 0(0) 0(0)	2(0) 3(1) 3(2)	4(3) 4(4) 4(3)	4(4) 4(4) 4(4)
29	3′-Hydroxy usnanilide		p	9 12 18	0 0 0	2 3 3	4 4 4	4 4 4
30	4′-Hydroxy usnanilide		p	9 12 18	4* 3 3	2 2 3	4 4 4	4 4 4
31	4′-Dimethyl amino-usnanilide		p	9 12 18	4 4 4	4 4 4	4 4 4	4 4 4
32	N,β-Carboxyethyl usnamide		s	10 15 20	1 2 1	3 4 4	4 4 4	4 4 4
33	*l*-usnamido napthalene-5-sulfonic acid		s	10 15 20	4 4 4	4 4 4	4 4 4	4 4 4
34	Vulpinic acid		s	10 15 20	1 2 2	3 3 3	4 4 4	4 4 4

* Suspension slightly turbid due to low solubility of drug. This may account for higher density readings.
† Samples of these were furnished by Prof. Alexander Robertson, F.R.S., to whom our grateful thanks are due.
‡ These differed in melting points from those previously recorded in literature, but gave correct analytical figures.
Solubility: s—solution, c—colloid, p—suspension.

might exhibit desirable solubility characteristics consisted in the condensation of usnic acid with substances bearing hydrophylic groupings. The compounds obtained in this connection are No. 29-33. Furthermore, a few substances possessing some of the structural features of usnic acid, No. 11-14, No. 19, No. 20, No. 22-27 have also been included for the examination of their antitubercular potentialities. A total of 33 compounds, related to usnic acid, have therefore been tested against the tubercle bacillus strain H37 Rv. in Dubos medium, using d-usnic acid for comparison. It appears desirable to report the results so far obtained in view of the recent publication of Shibata and Ukita on similar lines. They are given in Table I which also includes vulpinic acid.

We had hoped that this preliminary inquiry would reveal the minimum structural requirements of the usnic acid molecule essential for activity and the relationship, if any, between chemical constitution and antitubercular action in this group so that further planned progress could be made in the chemotherapy of mycobacterial infections. An examination of the present results, which are qualitatively in agreement with those of Stoll and his associates and Shibata and Ukita, could give rise to numerous generalities of a speculative nature. However, the relationship between structure and activity seems to be particularly obscure in this class of compounds. On the one hand, there is the utter lack of specificity of action of the stereoisomeric usnic acids unlike other optically and physiologically active natural products, and on the other, there is the apparently high degree of specificity of the *entire* usnic acid molecule for antitubercular activity. However, it is gratifying to find that some of the simple derivatives of usnic acid, the mono- and diacetyl usnic acids, dihydrousnic acid, m and p-hydroxyusnanilides, have activities equal to or comparable to that of the original substance. These compounds are now being subjected to further investigation.

[5] Dubos, R., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 361.

[6] Dubos, R., and Davis, B., *J. Exp. Med.*, 1946, **83**, 409.

Experimental. All tests were made by inoculating 0.1 ml of an 8-10-day-old culture diluted 6 x 10^3 into 5.9 ml of culture medium plus drug in 20 x 125 mm screw cap tubes which were then incubated at 37°C. Dubos liquid culture medium was used.[5,6] The culture tubes were previously selected to have the same optical density in the Coleman spectrophotometer to within 0.5%. Readings of the optical density of the culture in these tubes were then taken at 3, 10, 15, and 20 days after inoculation and compared with the growth of the control cultures grown at the same time.

All substances to be tested were dissolved in hot acetone, filtered thru a Corning U.F. filter into a small quantity of culture medium from which the acetone was then evacuated under reduced pressure. The sodium usnate was also tested by dissolving it in a minimal amount of warm (60°C) 2% Na_2CO_3 before addition to the culture medium. Both methods gave the same results. A few compounds gave clear solutions; the remainder were either colloidal or finely divided particulate suspensions as indicated in the table.

The optical density readings at 10, 15, and 20 days after inoculation were expressed as percent of the control readings and then classified into 4 groups, Group 1 being 1 to 25% of the control reading, Group 2, 25 to 50%, Group 3, 50 to 75% and Group 4, 75 to 100%. Zero indicates no growth as measured by optical density. The results are given in Table I. All tests were made in duplicate. In a few cases the tests were repeated at a later date. The values for these are indicated in parentheses.

Summary. The entire usnic acid molecule is involved in its action against the tubercle bacillus since any major alteration in the molecule markedly reduced or destroyed the activity. This is in marked contrast to the equal activity previously demonstrated for stereoisomeric usnic acids.

It is a pleasure to express our indebtedness to Professor Louis F. Fieser, in whose laboratories the chemical work was carried out, for his advice and encouragement, and also to Miss Blanche Burkhart for her assistance with the bacteriological tests.

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Site of Conversion of Tryptophan into Nicotinic Acid in Man.*

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Although direct evidence is now available for the conversion of tryptophan into nicotinic acid in the mammalian organism and of the intermediate steps concerned[1,2,3] the site of such conversion is still a matter of dispute. It is possible that different animal species behave differently in this regard, since there seem to be species differences[4–10] in the ability of tryptophan to spare nicotinic acid and in the proportion of excreted nicotinic acid derivatives. Studies to determine the site of the conversion are confined to experimental animals and have given contradictory results.[11]

Ellinger and Abdel-Kader[12] compared the oral and parenteral routes of administration in rats and observed a far greater rise in N^1 methyl nicotinamide output after oral administration. The magnitude of this response was markedly reduced by chemotherapeutic agents. This led them to conclude that the intestinal synthesis was of primary importance.

An opposite conclusion was reached by Schweigert and Pearson[13,14] who administered tryptophan both orally and intraperitoneally. The promptness of the response by the latter route, even though it was smaller in magnitude led them to believe that the conversion took place primarily in the tissues. This conclusion was somewhat modified in a later report by Junqueira and Schweigert[15] who studied the effect of succinyl sulfathiazole on the response to oral tryptophan with and without a pteroylglutamic acid supplement. In the presence of the sulfa drug when unsupplemented by pteroylglutamic acid, oral tryptophan had very little augmenting effect on N^1methyl nicotinamide excretion; but when pteroylglutamic acid was added the urinary excretion of the N^1methyl compound rose sharply.

In order to throw further light on this question we undertook to make comparisons of the effect of oral and intravenous tryptophan administration in infants.

Experimental. The subjects were 6 normal male infants, ranging in age from 3 to 24 months and in weight from 4.1 to 11.4 kg. They were maintained on standard formulae of evaporated milk and dextrimaltose through-

* This study was supported in part by grants from the Dazian Foundation for Medical Research, the Sugar Research Foundation, and Roche Anniversary Foundation.

† Mead Johnson Fellow in Pediatrics.

1 Albert, P. W., Scheer, B. T., and Deuel, H. J., Jr., *J. Biol. Chem.*, 1948, **175**, 479.

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5 Singal, S. A., Briggs, A. P., Sydenstricker, V. P., and Littlejohn, J. M., *J. Biol. Chem.*, 1946, **166**, 573.

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9 Luecke, R. W., McMillen, W. N., Thorp, F., Jr., and Tull, C., *J. Nutr.*, 1947, **33**, 251.

10 Schweigert, B. S., Pearson, P. B., and Wilkening, M. C., *Arch. Biochem.*, 1947, **12**, 139.

11 Perlzweig, W. A., Rosen, F., Levitas, N., and Robinson, J., *J. Biol. Chem.*, 1947, **167**, 511.

12 Ellinger, P., and Abdel-Kader, M. M., *Nature*, 1947, **160**, 675.

13 Schweigert, B. S., and Pearson, P. B., *J. Biol. Chem.*, 1947, **168**, 555.

14 Schweigert, B. S., and Pearson, P. B., *J. Biol. Chem.*, 1948, **172**, 485.

15 Junqueira, P. B., and Schweigert, B. S., *J. Biol. Chem.*, 1948, **175**, 535.

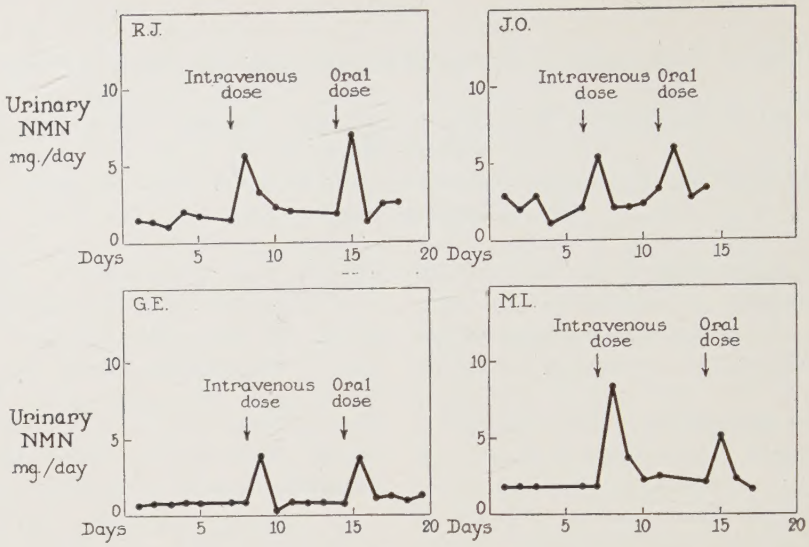

FIG. 1.
Effect of an intravenous and an oral dose of L-tryptophane on the urinary N¹methyl nicotinamide excretion.

out the period of study. After the urinary excretion of N¹methyl nicotinamide became fairly stable, the test dose of one gram L-tryptophan was administered either intravenously or orally.

The tryptophan solution for intravenous use was prepared in the following manner. One gram was dissolved in 30 cc of sterile distilled water containing 5 cc of 6 N hydrochloric acid, water was added to make a volume of 300 cc, and then the pH was adjusted with sodium hydroxide to neutrality. The solution was passed through a Seitz filter and then autoclaved for 10 minutes at 15 lb pressure. It was administered by slow drip, over a period of 3 to 6 hours. In 4 of the subjects the tryptophan was also administered orally, one week after the intravenous injection. The oral dose was divided in half and fed with the 10 A.M. and 2 P.M. bottles.

The N¹methyl nicotinamide determinations were performed according to the method of Huff and Perlzweig[16] on 24 hour collections of urine. The urines were collected in amber bottles with enough glacial acetic acid to make a 2% solution.

Results. The data for the 6 subjects are expressed graphically in Figs. 1 and 2. The slow intravenous administration of one gram of L-tryptophan resulted in a marked increase of urinary N¹methyl nicotinamide on the day of the infusion in 5 of these 6 subjects. In the sixth subject (V.C., Fig. 2), there was a moderate increase both on the day of as well as the day following the tryptophan injection. This slighter response can perhaps be attributed to the much larger size of this particular infant who received the same dose as the smaller subjects. We do not have an explanation for the delay in excretion.

In those subjects who also received the tryptophan orally, the rise in N¹methyl nico-

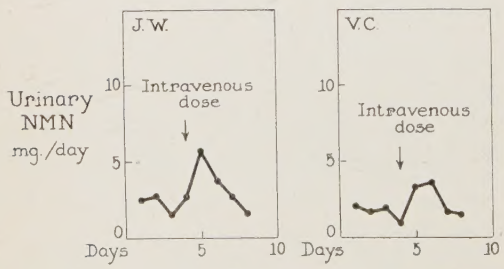

FIG. 2.
Effect of an intravenous dose of L-tryptophane on the urinary N¹methyl nicotinamide excretion.

[16] Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, 1947, **167**, 157.

tinamide excretion produced by this feeding was in the same range as that which resulted from the intravenous administration. Differences between the two routes of administration are within the limits of the usual daily variations that occur in the excretion of this substance. We have noted that the daily N^1 methyl nicotinamide excretion may fluctuate as much as 1.7 mg even when the infant is maintained on a constant diet.

Discussion. The rise of urinary N^1methyl nicotinamide excretion produced by the intravenous administration of L-tryptophan leads to the conclusion that the conversion of tryptophan to nicotinic acid in man takes place in the tissues rather than in the gastrointestinal tract as a result of bacterial action. This premise is strengthened both by the fact that the N^1methyl nicotinamide values are the same regardless of the route of administration, and by the fact that this increase takes place so promptly. For this conversion to occur as a result of tryptophan excretion into the gastrointestinal tract and subsequent action by bacteria there, one would anticipate a considerable time lag which actually did not occur.

The differences in the rates of administration are sufficient to explain the variance of our results from those of Schweigert *et al.*[13–15] One can postulate that our slow rate of injection raised the blood tryptophan level for a prolonged period of time thereby giving the tissues more time to produce the conversion. The method of a single large injection may provide such a large excess of tryptophan that it may be disposed of in other ways. This may also account for the results obtained by Ellinger and Abdel-Kader[12] who do not give any details regarding the technic of their parenteral tryptophan.

Of interest in this connection are the recent communications of Schweigert *et al.*[17] and of Singal, Sydenstricker and Littlejohn.[18] The former, working with chick embryos observed an increase in the nicotinic acid content of the tissues after an injection of tryptophan. The latter authors reported that the administration of L-tryptophan to rats caused an increase above normal of the nicotinamide concentration of the liver, a phenomenon observed in this organ alone.

Summary. The intravenous administration of one gram of L-tryptophan to infants on constant diets gave a prompt and large rise in the urinary N^1methyl nicotinamide which was equal in magnitude to that provoked by oral administration of the tryptophan. In view of these findings, the conversion of tryptophan to N^1methyl nicotinamide in man seems to be mediated by the body tissues rather than by bacterial synthesis in the gastrointestinal tract.

17 Schweigert, B. S., German, H. C., and Garber, M. J., *J. Biol. Chem.*, 1948, **174**, 383.

18 Singal, S. A., Sydenstricker, V. P., and Littlejohn, J. M., *J. Biol. Chem.*, 1948, **176**, 1069.

16997

Effect of Iodide on Thyroid Glands of Rats Kept at Low Temperature.

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A number of workers have noted that the thyroid glands of rats kept in the cold develop hyperplasia, thickened acinar epithelium, and loss of colloid characteristic of a hyperactive thyroid gland. It was of interest to determine whether the administration of iodide to rats kept under cold-room conditions would cause involution of the thyroid gland as it does in toxic goiter.

Four groups of male albino rats of the

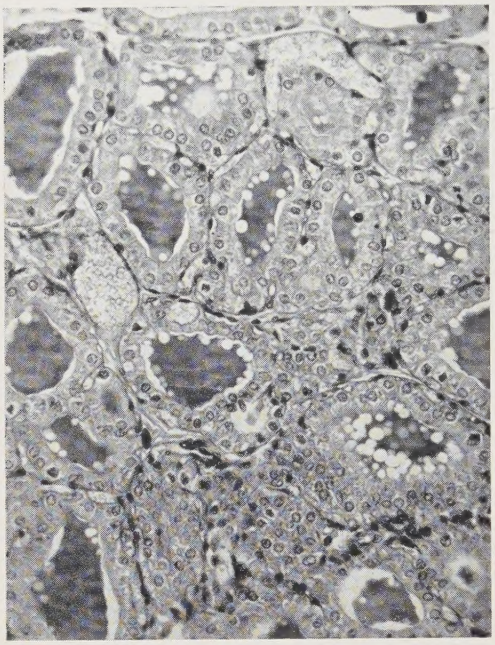

FIG. 1.

University of Southern California strain weighing between 200 and 350 g were used. Groups A and B were kept in single cages at room temperature (21 to 25°C) and groups C and D were kept in single cages with wire bottoms in a cold room maintained at 4°C. Groups A and C were on a stock diet consisting of ground oats 33%, cottonseed oil fortified with vitamins A and D 10%, wheat flour 38%, skimmed milk 14%, alfalfa 4%, yeast 2%, NaCl 0.5%, and $CaCO_3$ 0.5%. Groups B and D were maintained on this same diet to which had been added 50 mg KI per 100 g diet. The food intake and weight changes were determined at 3-day intervals.

At the end of 3 weeks, the animals were weighed, sacrificed, and the thyroid glands removed and weighed. The glands were fixed in formalin and stained with hematoxylin-eosin for histological examination. (In 6 cases animals were killed at the end of 2 weeks). The average epithelial heights and colloid diameters were determined on 100 different follicles from different parts of the thyroid glands.

Fig. I shows representative histological sections from thyroid glands of animals from the 4 groups. IA is typical of thyroid sections from Group A and shows a histological picture characteristic of a normal, moderately-active gland. 1B is a section from the thyroid gland of a rat from Group B receiving iodide at room temperature. It is seen that the gland is less active with thinner epithelium and more colloid. Exposure to low temperatures for 3 weeks brings about a marked hyperplasia with loss of colloid and thickened epithelium (1C). Administration of iodide prevented this hyperplasia and brought about a more normal-appearing thyroid gland in spite of the exposure to cold (1D).

Table I shows the data on the thyroid weights, epithelial heights, and colloid diameters of the 4 groups.

The data in Table I amplify the conclusions illustrated by Fig. 1 and show that iodide greatly reduces the thickness of the thyroid acinar epithelium and increases the colloid in the glands of animals made hyperactive by exposure to cold. These results are in accord with those obtained by Starr and Roskelley,[1] and stimulates the involuting effect of iodine in hyperthyroidism. It seems likely that iodine reduces the activity of the thyroid gland by a similar mechanism in both cases. It is of interest that the weight of the thyroid gland varied but little in the various groups—the amount of epithelium being inversely related to the amount of colloid.

Little can be said as to the mechanism of this involuting action of iodide at the present time. The work of Wolff and Chaikoff[2] clearly shows that increases in the blood iodide levels inhibit the formation of thyroxine by the thyroid gland of rats. Such an observation does not, however, explain the fact that iodide causes an accumulation of colloid in the thyroid follicles of cold-treated rats or of hyperthyroid individuals. It is possible that the effect of iodide on colloid accumulation may be brought about by a mechanism not related to hormone synthesis. The thyroid

[1] Starr, P., and Roskelley, R., *Am. J. Physiol.*, 1940, **130**, 549.

[2] Wolff, J., and Chaikoff, I. L., *J. Biol. Chem.*, 1948, **174**, 555.

TABLE I.
Effect of Iodide on the Thyroid Glands of Rats at Room Temperature or at 4°C.

Group	Treatment	No. of animals*	Wt gain	Thyroid weight, mg/100 g	Epithelial height, microns	Colloid diameter, microns§
A	Room temp. stock diet	5	10.8	10.4	9.1	41
B	Room temp. stock diet 0.05% KI	4	—26.3	12.7	3.5	60
C	4°C stock diet	11†	—18	12.8	12.9	34
D	4°C stock diet 0.05% KI	5‡	—43	13.4	8.3	47

* No. of experimental animals varies due to deaths during the course of the experiment. Figures represent animals surviving at end of 21 days.
† Including 4 animals killed at end of 2 weeks.
‡ Including 2 animals killed at end of 2 weeks.
§ Mean of long and short diameters.

develops like a salivary gland which later loses its excretory pathways, leaving the thyroglossal duct as a remnant. Colloid may, therefore, be considered as a secretory product of the thyroid gland trapped in the alveoli. Iodide is known to cause hypersecretion and hypersalivation and may therefore also be considered to induce secretion of colloid into the thyroid follicles. Such colloid would, from the results of Wolff and Chaikoff, be expected to be low in organic iodine.

Summary. The hyperplasia of the thyroid epithelium produced in rats by exposure to cold-room conditions for 3 weeks can be prevented by increasing the iodide level of the diet. The mechanism of this effect would appear to correspond to the involuting action of iodide in the thyroids of patients with hyperthyroidism. It is difficult to explain these effects on the basis of the inhibition of hormone synthesis, and it is submitted that iodide may stimulate the secretion of colloid into the lumen of thyroid follicles by a mechanism related to its stimulating influence on salivation.

16998

Tetanus Prophylaxis with Penicillin-Procaine G.

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Clostridium tetani is sensitive to penicillin *in vitro*[1] and *in vivo*.[2,3] The development of penicillin-procaine has resulted in prolonged therapeutic blood levels following a single injection.[4,5] Claims have been made for inhibitory levels which are maintained for as

1 Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, E. D., Heatley, N. G., Jennings, M. A., Florey, H. W., *Lancet*, 1941, **2**, 177.

2 Weinstein, L., and Wesselhoeft, C., *New England J. Med.*, 1946, **233**, 681.

3 Diaz-Rivera, R. S., Deliz, L. R., Berio-Suarez, J., *J.A.M.A.*, 1948, **138**, 191.

4 Herrell, W. E., Nichols, D. R., and Heilman, F. R., *Proc. Staff Meet., Mayo Clinic*, 1947, **22**, 567.

5 Sullivan, N. P., Symmes, A. T., Miller, H. C., Rhodehamel, H. W., Jr., *Science*, 1948, **107**, 169.

TABLE I.
Mortality in Penicillin-Treated Mice Infected with *Cl. tetani.*

Series	Units of penicillin	Hours before penicillin	Total animals	Survivors	Time to death (days)	Mortality, %
A	0	0	32	1	<4	96.8
B	150	0	25	20	7-10	20.0
C	150	0	25	5	<5	80.0*
D	300	0	25	21	4- 8	16.0
E	150	3	25	21	7-10	16.0
F	150	6	18	14	6-10	22.2
G	150	24	19	9	<5	52.6

* Penicillin injected IM in opposite leg rather than infected leg.

long as 4 or 5 days following injection of 300,000 or 600,000 units.

The possibility of penicillin action against spores of tetanus in tissue during the incubation period of the disease posed the probability of its use as a prophylactic agent. Its value in this instance would be especially desirable in minor injuries where there is hesitancy in the use of antitoxin because of the dangers from foreign protein sensitization. Its use to prevent toxin formation in tissue would seem more rational than use of antitoxin to neutralize toxin after it is formed.

The results obtained with mice as shown in data presented demonstrate a definite prophylactic effect. More detailed evidence will be presented later.

*Methods. Clostridium tetani** was grown in Trypticase Soy Broth with added fresh calf brain at 37°C for about 12 days, then filtered through sterile cheesecloth to remove meat particles, centrifuged and resuspended in sterile distilled water in 8 oz. screw-capped bottles. Toxin and vegetative cells were destroyed by heating in a water bath for 30 minutes at 80°C. Spore suspensions were then shaken vigorously 30 times to suspend spores evenly, and counted by adding serial dilutions in duplicate to melted and cooled Trypticase Soy Broth (1.5% agar added) deeps. After solidification a one-half inch layer of 2% stratifying agar was poured on top to insure adequate anaerobiosis. The appearance of macroscopic colonies upon incubation yielded a count only of viable spores. Spore suspensions were then adjusted to 1,000,000/ml. and stored at 4°C.

* Courtesy of Parke Davis and Company, Detroit, Mich.

Since it has been adequately demonstrated that the spores of *Cl. tetani* are not infective in healthy tissue, $CaCl_2$ in 5% solution was used as a tissue debilitant.[6-8]

The number of spores necessary to produce approximately 100% mortality in White Swiss mice was determined by injecting mice with 0.1 ml 5% $CaCl_2$ immediately followed by 10, 100, 1,000, 10,000, and 100,000 spores of *Cl. tetani* intramuscularly in the inner surface of the left hind leg. Mice receiving 10 and 100 spores survived; those receiving 1,000 spores died with symptoms of tetanus in 3-5 days, and with 10,000 and 100,000 spores, 2-3 days. Thus 1,000 spores was chosen as an LD_{100} infective dose.

To determine the possible prophylactic value of penicillin in tetanus, Penicillin G Procaine in oil with 2% aluminum monostearate,† containing 300,000 units/ml was used. The contents of one disposable cartridge containing 300,000 units were added to 99 ml sterile sesame oil and shaken 30 times before each usage to procure a homogenous suspension. With this dilution 0.1 ml contained 300 units penicillin, 0.05 ml contained 150 units. (The latter is comparable to 600,000 units for an adult human being.) Injections of penicillin were made in the same leg used for the infective dose of spores and $CaCl_2$, except in one series (Table I, Series C) where the opposite leg was used.

The time lapse between the injections of

6 Bullock, W. E., and Cramer, W., *Proc. Royal Soc. London*, 1919, B, **90**, 513.

7 Russell, D. S., *Brit. J. Exp. Path.*, 1927, **8**, 377.

8 Fildes, P., *Brit. J. Exp. Path.*, 1927, **8**, 387.

† Supplied by Abbott Laboratories, North Chicago, Ill.

TABLE II.
Statistical Analysis of Data on Mortality and Time to Death.

Series	Mortality		Time to death		
			Days		
	%	P value*†	Mean	S.D.	P value*‡
A	96.8	—	3.31	1.42	—
B	20.0	<.01	8.40	3.47	<.01
C	80.0	>.10	3.90	1.69	>.05
D	16.0	<.01	6.25	2.51	<.01
E	16.0	<.01	7.75	2.94	<.01
F	22.2	<.01	6.75	3.07	<.01
G	52.6	<.01	4.10	2.11	>.05

* Series compared with Series A.
† P values taken from Chi-square 4-fold table including Yates correction; value less than 0.05 indicates significant difference.
‡ P values taken from Fisher's "t" table; value less than 0.05 significant.
S.D., Standard Deviation.

the infective dose and penicillin was varied, since other reports on the clostridia[9–15] indicate that elapsed time is an important factor in protection.

White Swiss mice of 15 to 35 g weight were used with a holding period of 10 days after the initial infective dose. Results after this interval are shown in Table I.

A statistical analysis of data[16] on mortality and time to death of mice receiving penicillin prophylaxis and untreated controls is presented in Table II.

The P value (likelihood of difference arising through chance alone) of the mortality of each series as compared with controls (Table II, Series A) was obtained from a Chi-square test of independence 4-fold table including Yates correction. P values of series B, D, E, F, and G (Table II) are significant.

The statistical analysis of the difference in time to death in days between each series and series A includes the mean time to death in days and the standard deviation with the P value obtained from Fisher's table indicating the significance of the data. Series B, D, E, and F have significant values.

Discussion. Both the reduction in mortality and prolongation of life of treated mice indicate the prophylactic effect of the penicillin-procaine compound. Administration of penicillin within 24 hours after the injection of spores of *Cl. tetani* serves to reduce the mortality as compared with the untreated controls. The lowest mortalities resulted when 150 and 300 units of penicillin were given in the infected leg immediately, and when 150 units were given after 3 and 6 hours' delay (Table I, Series B, D, E, and F). These series having low mortalities were also observed to have the greatest time lapse before symptoms and deaths. When compared to the rapid completion of the disease as observed in the controls (Table I, Series A), the noticeably longer time required before symptoms and deaths occur in the low mortality series receiving penicillin (Table I, Series B, D, E, and F) may indicate that the number of organisms capable of producing toxin is considerably reduced and that only when there are organisms surviving in remaining necrotic areas, which multiply and produce a lethal dose of toxin, does a fatality result. The similarity between the mortality rates

9 Chain, E., Florey, H. W., Gardner, A. D., Heatley, N. G., Jennings, M. A., Orr-Ewing, J., and Sanders, A. G., *Lancet*, 1940, **2**, 226.

10 McIntosh, J., and Selbie, F. R., *Lancet*, 1942, **2**, 750.

11 Dawson, M. H., Hobby, G. L., Meyer, K., and Chaffee, E., *Ann. Int. Med.*, 1943, **19**, 707.

12 McKee, C. M., Hamre, D. M., and Rake, G., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 211.

13 McIntosh, J., and Selbie, F. R., *Lancet*, 1943, **2**, 224.

14 Hac, L. R., and Hubert A. C., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 61.

15 Hac, L. R., *J. Inf. Dis.*, 1944, **74**, 164.

16 Snedecor, G. W., *Statistical Methods*, Ames, Iowa, Iowa State College Press, 1946, 485 pp.

and time lapse before death in series B, where an immediate injection of penicillin was given, and in series E and F where administration of penicillin was delayed for 3 and 6 hours respectively, may be explained by the fact that it is generally acknowledged that penicillin is most effective against sensitive microorganisms during the period of active growth and multiplication; therefore the efficacy of penicillin administered after 3- or 6-hour lapses may indicate that those times allow germination of spores[8] and the vegetative cells are then inhibited by the concentration of penicillin available. Since Penicillin G Procaine in oil is reported to maintain concentrations at effective therapeutic levels for 96 hours, the longer time lapse before symptoms appear may be accounted for if it is only after this level has dropped that a lethal toxin is produced by survivors.

Higher mortalities were observed in series C and G. When 150 units of penicillin was administered with no delay but in the leg opposite the necrosis, 80% of the mice died (Table I, Series C). When the penicillin was injected into the necrotic leg after a 24-hour delay, 52.6% of the mice died (Table I, series G). The indication in series C is that (1) a sufficient amount of penicillin does not reach the necrotic area to inhibit growth and production of toxin by *Cl. tetani,* with resultant death of the animal, or (2) that organisms produce a lethal dose of toxin before being inhibited, with this latter possibility being especially applicable to series G. The rate at which penicillin is released and the rate and extent of penetration into necrotic areas are probably the significant factors. The similarity of series C and G to series A (Table I) in the short time lapse before death indicates that toxin production had taken place rapidly, and if series G is compared with series F this observation is further verified since the additional 18 hours time lapse before penicillin was administered caused a 30% increase in mortality.

Conclusions. Results indicate that penicillin-procaine G in sesame oil and 2% aluminum monostearate is of significant value prophylactically in lowering the mortality of mice experimentally infected with a lethal dose of detoxified spores of *Clostridium tetani.* In addition to decreasing the mortality, it retards the development of symptoms and resulting deaths. Injected into the necrotic areas, it is more effective than the same unitage injected at an uninfected site. Whether this is caused by insufficient penetration of the drug due to the presence of necrotic tissue or to interference by the calcium chloride has not been determined.

16999

Further Observations on Isosensitization to the Rh Factor.

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The purpose of this report is to describe the results of experiments on Rh sensitization in man. The findings are of significance in relation to the pathogenesis of intragroup hemolytic transfusion reactions,[1,2] and the pathogenesis of erythroblastosis fetalis,[3] as well as the practical problem of producing Rh testing sera.[4]

The subjects used in these experiments were 47 adult male individuals. Each individual was subjected to a series of injections of group

[1] Wiener, A. S., and Peters, H. R., *Ann. Int. Med.,* 1940, **13**, 2306.

[2] Unger, L. J., and Wiener, A. S., *Am. J. Clin. Path.,* 1945, **15**, 280.

[3] Wiener, A. S., *Am. J. Dis. Child.,* 1946, **71**, 14.

[4] Wiener, A. S., and Gordon, E. B. S., *Am. J. Clin. Path.,* 1947, **17**, 67.

TABLE I.
Observations on Rh Sensitization in Males.

No. of inj.	No. of subjects	Frequency of Rh antibody formation		% of total series first showing Rh antibodies at inj. in question	Cumulative total (%)	
		No.	%		Sensitized	Not sensitized
2	47	18*	38.3	38.3	38.3	61.7
3	23	6	26.1	26.1 × 0.617 = 16.1	54.4	45.6
4	15	3	20.0	20.0 × 0.456 = 9.1	63.5	36.5
5	10	1	10.0	10.0 × 0.365 = 3.7	67.2	32.8
6	3	1	33.3	33.3 × 0.328 = 10.9	78.1	21.9

* One subject produced Rh antibodies after the very first injection. In this case a history was elicited of several blood transfusions received many years previously during treatment for osteomyelitis.

O, Rh_0-positive blood, each dose consisting of 2 to 4 cc of a 50% suspension of whole blood in sodium citrate solution. The injections were given at intervals of 3 to 4 months. All the injections were given intravenously, except that occasionally part of the blood was deposited into or under the skin. Tests for Rh antibodies were made in the usual manner[5,6] by the agglutination and albumin-plasma conglutination methods. The antibody tests were carried out 10 to 14 days after each injection, except that in most cases no tests were done after the very first injection. According to our previous observations,[2,4] as a rule a minimum of 2 injections, spaced 3 to 4 months apart, are needed to induce the formation of Rh antibodies, the first injection acting as a primer and the second or subsequent injection stimulating the production of specific antibodies.

The results are summarized in Table I. It will be seen that after the second injection, 18 of the 47 subjects, or 38.3%, had demonstrable antibodies in their sera. One of these 18 subjects had showed antibodies after the very first injection, but after careful questioning a history was elicited of previous blood transfusions many years before when this individual had been treated for osteomyelitis. Of the 29 subjects who failed to respond to the second injection, 23 returned for a third injection, and 6 of these or 26.1% then showed antibodies. If we assume that the 6 individuals who did not return for the third injection had an equal chance of becoming sensitized, then this indicates that the third injection would sensitize 26.1% of the 61.7% of the nonsensitized individuals remaining after the second injection, or 16.1% of the total number injected. By adding this 16.1% to the 38.3% representing the individuals sensitized by the second injection, we find that fully 54.4% of the Rh-negative individuals became sensitized after a course of 3 properly spaced injections of Rh-positive blood. Of the 17 individuals who failed to respond to the third injection, 15 returned for a fourth injection, and of these 3 individuals or 20% became sensitized. As shown in the table, this raises to 63.5% the total incidence of sensitization after a course of 4 injections; and similarly, the percentage of sensitized individuals is raised to 67.2% by a fifth injection of Rh-positive blood. Unfortunately, only 3 subjects returned for the sixth injection, of whom one became sensitized. Obviously, this result has a large statistical error and should therefore be discounted. Judging from the results of the third, fourth, and fifth injections, it would appear as if the percentage of sensitized individuals was approaching a limit somewhere between 70 to 80%; while if the results of the sixth injection could be taken literally, it would seem that all the injected individuals would eventually become sensitized following a sufficient number of injections of blood. This question can obviously only be settled by experiments on a larger series of individuals, over a longer course of injections.

In a parallel series of experiments, a number of type Rh_1Rh_1 individuals was subjected

[5] Wiener, A. S., *Am. J. Clin. Path.*, 1946, **16**, 477.

[6] Wiener, A. S., and Hurst, J. G., *Exp. Med. and Surg.*, 1947, **5**, 285.

to a series of injections of type rh blood in an attempt to induce sensitization to the factor hr′ or Hr_0, or both. Nineteen of these individuals received 2 injections; of these 13 received a third injection, 8 received a fourth injection, 5 received a fifth injection, while 4 received a sixth injection. Not one of the individuals of this series showed any Hr antibodies at any time. This, despite the smaller number of individuals injected, is in sharp contrast to our experiences with sensitization against Rh_0 factor. Moreover, these results conform with the conclusions based on clinical observations that the Hr factors are much weaker antigens than the Rh factors.[7]

Summary. The results of experiments on sensitization of male type rh individuals by intravenous injections of Rh_0-positive blood at intervals of 3 to 4 months are described. Of 47 subjects receiving 2 injections, 38.3% became sensitized; after 3 injections, the incidence of sensitization rose to 54.4%; after 4 injections, the percentage rose to 63.5%, while after 5 injections the incidence of sensitization was 67.2%. Because of the small number of individuals receiving more than 5 injections, it is not possible to state with certainty whether the incidence of sensitization approaches a maximum limit somewhere between 70 and 80% of individuals injected, or whether all individuals eventually would become sensitized providing they are given a sufficient number of injections. In any event, it is obvious that the deliberate injection of Rh-positive blood is a potent means of sensitizing Rh-negative individuals.

In contrast to these results, not one among 19 type Rh_1Rh_1 individuals receiving repeated injections of type rh blood became sensitized to the Hr factors. These results confirm the conclusion based on clinical observations that the Hr factors are much weaker antigens than the Rh factors.

The author wishes to express his appreciation to Miss Helen Reiss, who assisted with the blood injections, and to Mrs. E. B. Gordon, Mrs. L. Katz, and Mrs. C. Mazzarino for their assistance in carrying out the Rh antibody tests. He is also indebted to Mr. I. H. Gilbert of the Certified Blood Donor Service for his cooperation in providing the blood donors who submitted to the blood injections.

[7] Wiener, A. S., *J. Lab. and Clin. Med.*, 1948, **33**, 985.

17000

Glycolysis in *Plasmodium gallinaceum.**

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For many years a practical method of cultivating the malaria plasmodia has been sought, and recently one has been devised by Ball and his colleagues[1,2] for the erythrocytic stages of *Plasmodium knowlesi*, a parasite of monkeys. Monkeys, however, are expensive and much less easily maintained in the laboratory than birds, and for this reason it is important that a good culture technic be devised for the avian plasmodia,‡ which are equally suited for the screening of antimalarial drugs and for other types of research in malaria. It

* Aided by a grant from the National Institute of Health.

† Present address of the junior author, Dept. of Biochemistry, University of Wisconsin.

[1] Ball, E. G., Anfinsen, C. B., Geiman, Q. M., McKee, R. W., and Ormsbee, R. A., *Science*, 1945, **101**, 542.

[2] Geiman, Q. M., Anfinsen, C. B., McKee, R. W., Ormsbee, R. A., and Ball, E. G., *J. Exp. Med.*, 1946, **84**, 583.

‡ Hawking[3] has devised a tissue culture technic for growing the exoerythrocytic stages of several species of avian plasmodia.

[3] Hawking, F., *Trans. Roy. Soc. Trop. Med. and Hyg.*, 1945, **39**, 245.

is also clear that an integrated picture of the malaria problem cannot be had until our knowledge includes as much about the non-human species of malaria as it does of the plasmodia of man.

For these reasons, the authors attempted to adapt the Ball culture method to *Plasmodium gallinaceum*, the parasite of chicken malaria, but with only partial success, and it then seemed evident that some careful studies of its biochemistry would be necessary before greater success could be hoped for. Since the addition of more glucose to the medium than originally called for seemed to improve its performance somewhat, a detailed and quantitative study of glycolysis in this species was undertaken.¶

Materials and Methods. The strain of *Plasmodium gallinaceum* used was obtained from Dr. Gilbert Otto of the Johns Hopkins University School of Hygiene and Public Health. Inoculation of the chicks was done either intraperitoneally or intramuscularly with brain suspensions or parasitized blood, usually when they were about three weeks old. Blood for experimental use was obtained by intracardial puncture, without anesthesia, to insure normal blood sugar levels. To avoid possible effects of a developing immunity, chicks were used during the period of rising parasitemia. The anticoagulant was Lederle heparin.

After removal from the bird the blood was taken immediately to the constant temperature (40°C) room, 0.5 ml was saved for immediate examination, and the balance placed in a rocker dilution tube, equilibrated with 5% CO_2 and 95% air, and rocked according to the Ball cultivation technic, samples being taken at regular intervals for glucose determinations, which were always made in duplicate. These were plotted against time and the hourly rate of glucose consumption calculated. This last was done per ml of blood, both normal and parasitized, and per mature red cell, reticulocyte, and parasite.

Glucose levels were determined by the Folin-Malmros method (Klett-Summerson Clinical Manual), except that a ferric duponal solution[4] was substituted for the ferric gum-ghatti solution originally specified, thus avoiding the partial precipitation of the Prussian blue which sometimes occurred with the gum ghatti. The glucose values usually differed from their mean by no more than ± 3 mg %. Erythrocyte and parasite counts were made on each sample, enough parasites being counted to keep the error within 10%[5] Differentials were also made. The stain used was the J.S.B.[6]

Whole undiluted blood was employed for all determinations. For the study of the glucose consumption of reticulocytes, chicks were made anemic by 3 previous daily injections of phenylhydrazine hydrochloride.

Results. The hourly glucose consumption rates of normal chicken blood and of blood containing a high proportion of reticulocytes (79.5%) are illustrated in Fig. 1 and 2 respectively. Fig. 3 shows in similar fashion the hourly glucose consumption of parasitized blood. With larger numbers of parasites, or greater proportions of reticulocytes, curves with even steeper initial slopes would be obtained.

Five determinations were made of the hourly glucose consumption of unparasitized

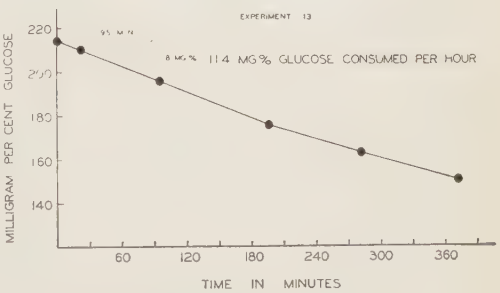

FIG. 1.

Glucose consumption of unparasitized blood containing approximately 100% adult red cells.

¶ Since the writing of this report, the paper by Marshall (*Brit. J. Pharm.*, **3**, 1) has come to our attention. Although his determinations of oxygen uptake of parasitized blood indicate glycolysis values for *P. gallinaceum* essentially like ours, added glucose failed to stimulate respiratory activity.

4 Klenshoj, N. C., and Hubbard, R. S., *J. Lab. and Clin. Med.*, 1939, **25**, 1102.

5 Hartman, E., *Am. J. Hyg.*, 1927, **7**, 407.

6 Singh, J., and Bhattacharji, L. M., *Ind. Med. Gaz.*, 1944, **79**, 102.

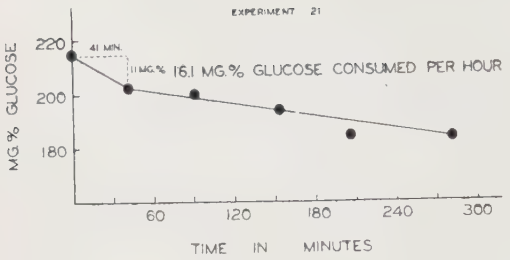

FIG. 2.

Unparasitized blood containing approximately 20.5% adult red cells and 79.5 reticulocytes.

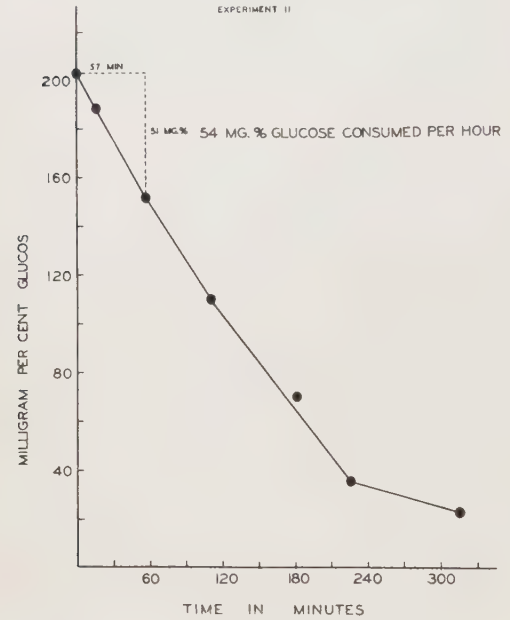

FIG. 3.

Glucose consumption of parasitized blood, containing 89.5% adult red cells, 10.5% reticulocytes, and 178 parasites per 100 red cells (Type A, 59%, Type B 36%, and Type C 5%).

blood, 3 of these on normal and 2 on highly anemic samples. The results were 0.114, 0.064, and 0.083 mg per ml on the former, and 0.161 and 0.360 mg per ml on the latter. From these figures the hourly glucose consumption per 10^9 red cells (for both mature erythrocytes and reticulocytes) was calculated.‖ The mean value for mature erythrocytes was found to be 4.34×10^{-2} mg and for reticulocytes 3.06×10^{-1}. Thus the average mature red cell has a metabolic rate only about one-seventh that of a reticulocyte.

The hourly glucose consumption rate of parasitized cells is much higher, although it depends on the size and stage in the asexual cycle of the parasite. Five determinations were made of the glucose consumption of parasitized blood. (Table I) The mean value, in mg per hour per ml, was 0.48—a figure substantially higher than any of the rates obtained for unparasitized blood, normal or anemic.

To make such values really comparable with those obtained for unparasitized blood it is necessary to know both the parasite count, and the proportions present of parasites of different sizes. Silverman and his colleagues[7] distinguished 3 types. Merozoites freshly formed by segmentation they called "Type A." Somewhat larger stages, having up to 4 masses of chromatin, they designated as "Type B," and all others were called "Type C." They found the surface areas of these 3 types to be approximately 1, 12, and 48 square micra.

Using this scheme of classification, we determined their hourly glucose consumption rates to be about 3.00×10^{-11} mg, 3.60×10^{-10} mg, and 1.44×10^{-9} mg respectively. An "average" parasite (one having the mean of these 3 areas) consumed glucose about 14 times as fast as an adult erythrocyte and twice as fast as a reticulocyte. The data on which these and other calculations were based is summarized in Table I.

From these data the glucose consumption rates per square micron of surface area may also be calculated, both for erythrocytes and parasites. We found this to be 3.42×10^{-13}

‖ This involved the use of the formula

$$\text{mg glucose per hour per ml} = E(AX \times RX')$$

in which

$$A = \frac{\text{adult erythrocytes}}{\text{total erythrocytes}}$$

$$E = \frac{\text{erythrocytes per mm}^3}{10^6}$$

$$R = \frac{\text{reticulocytes}}{\text{total erythrocytes}}$$

X = hourly glucose consumption (mg) of 10^9 red cells

X′ = hourly glucose consumption (mg) of 10^9 reticulocytes

[7] Silverman, M., Ceithaml, J., Taliaferro, L. G., and Evans, E. A., *J. Inf. Dis.*, 1944, **75**, 212.

TABLE I.
Summary of Experimental Data.*

A. Normal Unparasitized Blood.

Experiment	E	A	R	Mg glucose per hr per ml blood	X
13 (Fig. 1)	1.91	1.000	0.000	0.114	5.97×10^{-2}
14	1.86	0.995	0.005	0.064	3.36×10^{-2}
15	2.25	1.000	0.000	0.083	3.68×10^{-2}
				Mean:	4.34×10^{-2}

B. Anemic Unparasitized Blood.

Experiment	E	A	R	Mg glucose per hr per ml blood	X′
21 (Fig. 2)	0.67	0.205	0.795	0.161	2.91×10^{-1}
22	1.58	0.335	0.665	0.360	3.21×10^{-1}
				Mean:	3.06×10^{-1}

C. Parasitized Blood.

Experiment	E	A	R	P	a	b	c	Mg glucose per hr per ml blood	Mg glucose per hr per 10^7 μ^2 par. area
11 (Fig. 3)	1.33	0.895	0.105	178	59	36	5	0.54	2.57×10^{-4}
12	1.81	0.990	0.010	61	15	76	9	0.42	2.27×10^{-4}
16	0.78	0.650	0.350	200	4	93	3	0.75	3.24×10^{-4}
18	1.87	0.810	0.190	10.5	18.5	16.7	64.8	0.35	2.66×10^{-4}
19	1.34	0.562	0.438	19.8	0.0	98	2	0.36	4.27×10^{-4}
								Mean:	3.00×10^{-4}

* Abbreviations used in Table I, and in the formulae:
a, b, c = percentage distribution of 3 parasite types

$$E = \frac{\text{erythrocytes per mm}^3}{10^6 \text{ adult erythrocytes}} \qquad R = \frac{\text{reticulocytes}}{\text{total red count}} \qquad X = \text{mg glucose per hr per } 10^9 \text{ red cells}$$

$$A = \frac{\quad}{\text{total red cell count}} \qquad P = \text{parasites per 100 red cells} \qquad X' = \text{mg glucose per hr per } 10^9 \text{ reticulocytes}$$

TABLE II.
Glucose Consumption (mg per hour).

Authority	Erythrocytes (per red cell)	Erythrocytes (per μ^2 of area)	Parasites (per μ^2 area)	Ratio (col. 2/col. 3)
Our work	4.34×10^{-11}	3.42×10^{-13}	300×10^{-13}	1/87.8
Silverman *et al.*	6.25×10^{-12}	4.92×10^{-14}	390×10^{-14}	1/79.0
Ratio (our values to Silverman's)	6.95/1		7.7/1	

mg per hour for normal erythrocytes (adult), and 300×10^{-13} for parasites. Both values greatly exceed those of Silverman and colleagues (Table II), and we believe the differences are probably due to the different treatment given the blood in our work, since we used no diluting agent and did not wash the cells before making the tests, thus keeping the cell environment more nearly physiologically normal.

To make these calculations, and also to predict the glucose consumption of cultures containing any given number of parasites, the following formula was developed:

$$W_t = 4.34E \times 10^{-2} (A + 7.05R + 6.92P \times 10^{-5} (a + 12b + 48c))$$

in which

W_t = mg glucose consumed per hour per ml of parasitized blood
a, b, and c = percentage distribution of 3 parasite types
A, E, R, X, and X′ are as indicated above (formula used in computing glucose consumption of unparasitized blood)

The formula is easily derived, assuming

that the glucose consumed by parasitized blood (W_t) is the sum of that utilized by adult red cells (W_A), reticulocytes (W_R), and parasites (W_P) in unit time.

W_A = number of red cells in 1 ml blood × hourly glucose consumption of 1 red cell
or $(10^9 \times A \times E) \times (4.34 \times 10^{-11}) = 4.34AE \times 10^{-2}$ mg

W_R = number of reticulocytes in 1 ml blood × hourly glucose consumption of 1 reticulocyte
or $(10^9 \times R \times E) \times (3.06 \times 10^{-10}) = 3.06RE \times 10^{-1}$ mg

W_P = total square micra of parasite surface area × hourly glucose consumption per square micron
or $(PE \times 10^5 (a + 12b + 48c)) \times (3.00 \times 10^{-11}) = 3.00PE \times 10^{-6} (a + 12b + 48c)$ mg

Therefore

$W_t = (4.34AE \times 10^{-2}) + (3.06RE \times 10^{-1}) + (3PE \times 10^{-6}) (a + 12b + 48c) = 4.34E \times 10^{-2} (A + 7.05R + 6.92P \times 10^{-5} (a + 12b + 48c))$

Discussion. In making the calculations and constructing the formula above it has been assumed that (1) the blood cells are in an essentially normal environment for at least the first 30 minutes, and therefore metabolize normally, (2) the metabolism of leucocytes and thrombocytes is normal and approximately constant in each sample, (3) the levels of any hormones influencing red cell metabolism are constant, or negligible in effect, (4) the metabolism of the infected red cell is undisturbed by the presence of the parasite, and (5) the metabolism of the gametocytes is the same, or not greatly different from that of asexual forms of like size. The fourth and fifth assumptions may be open to question, but it has so far not been possible to put them to experimental test.

Despite certain sources of error inherent in the experimental procedures, such as the counting technics, agreement between the experimentally determined and the values predicted by the formula has been close, the mean deviation between the two having been only ± .036 mg glucose per ml per hour.

Summary and Conclusions. A study of the glucose requirements of *Plasmodium gallinaceum* has been made, and formulae derived for predicting the hourly glucose consumption rates of parasitized and unparasitized blood. These should be useful in predicting the amounts of glucose required for cultures and indicative of the *in vivo* drain on carbohydrate stores. Normal chicken blood was found to have an hourly glucose consumption rate of approximately 8.7 mg per 100 ml, and values as high as 74.7 mg per 100 ml were observed for parasitized blood. The glucose consumption per square micron per hour was 3.42×10^{-13} mg, 2.41×10^{-12} mg, and 3.00×10^{-11} mg for adult red cells, reticulocytes, and parasites respectively.

17001

Riboflavin and Growth of Tubercle Bacilli.

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It was reported previously (Hou;[1] Farber and Miller[2]) that riboflavin deficiency was common among patients suffering from tuberculosis and that phlyctenular conjunctivitis or keratitis frequently observed in tuberculous patients responded to riboflavin therapy (Hou[3]). It was postulated (Hou[1]) that although tubercle bacilli will grow in the ab-

* On leave from The Institute of Nutrition, China, and on a traveling fellowship of the World Health Organization of the United Nations.

1 Hou, H. C., *Chinese J. Med.*, 1943, **62**, 181.

2 Farber, J. E., and Miller, D. K., *Am. Rev. Tuberc.*, 1943, **48**, 412.

3 Hou, H. C., *Chinese Med. J.*, 1940, **58**, 616.

TABLE I.
Influence of Riboflavin on the Growth of Tubercle Bacillus.

Medium, character of	% transmission with air blank as reference			
	Control, no riboflavin	Riboflavin, % conc.		
		0.25	0.5	1.0
Vit. free	68.5	66.4	67.1	66.3
+ Vit. A	61.8	61.1	—	—
+ Vit. A + Biotin	58.4	61.4	—	—
+ Vit. A + Thiamine	58.8	58.6	—	—
+ Vit. A + Biotin + Thiamine	59.3	57.2	—	—

sence of riboflavin (Boissevain *et al.*[4]; Street and Reeves[5]) they may remove riboflavin from the host during the multiplication and thus bring about a deficiency condition. The present experiment was carried out to determine first whether the addition of riboflavin to the medium would promote the growth of tubercle bacilli and second whether there would be a loss of riboflavin from the medium during the growth of the organism.

Procedure. Human tubercle bacilli of a slightly virulent strain TBI† were used. The culture medium was made according to the formula of Dubos and Middlebrook[6] with the following modifications: Twenty ml of hydrolyzed vitamin free casein solution (1 ml corresponds to 100 mg casein) were used instead of 2 g enzymatic digest of casein. Tween 80 was purified according to the method of Davis[7] while bovine albumin was omitted.

For the study of the effect of riboflavin on the growth of tubercle bacillus 10 ml portions of the medium were distributed in 50 cc Erlenmeyer flasks. For each series 4 to 6 flasks were used as controls while a similar number was used for each of 3 different levels of riboflavin, namely, 0.025 mg, 0.05 mg, and 0.1 mg per 10 ml. After the addition of riboflavin or other vitamin solutions, the final volume of liquid in each flask was made up to the same amount by the addition of sterile distilled water. To prevent the destruction of riboflavin by light the flasks containing the riboflavin solution were covered with black cloth or paper. All flasks containing the medium were incubated for 24 hours to see if any was contaminated. One-tenth ml of a liquid tubercle bacillus culture 7 to 10 days old was added to each flask. The amount of bacterial inoculum was estimated to be 0.0014 mg, 0.014 mg and 0.14 mg for different series of experiments. After 4 to 7 days incubation at 37°C the degree of growth was determined turbidimetrically by means of a Coleman Junior Spectrophotometer. The metal screw-capped test tubes used for the reading were read first with air as a reference. The wave length was set at 560 mμ.

For the study on the loss of riboflavin from the medium, 4.0 mg of riboflavin in 80 ml of 0.02 N acetic acid solution were added to 1 liter of medium so that each ml of the medium contained about 4 γ of riboflavin. One hundred ml were distributed in each of 10 flasks, which were protected from light by wrapping with black cloth or paper. After incubation for 24 hours, to determine if the medium re-

4 Boissevain, C. H., Drea, W. F., and Schultz, H. W., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 481.

5 Street, H. R., and Reeves, R. F., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 641.

† Originally came from the Bureau of Animal Industry.

6 Dubos, R. J., and Middlebrook, G., *Am. Rev. Tuberculosis*, 1947, **54**, 334.

7 Davis, B. D., *Arch. Biochem.*, 1947, **15**, 359.

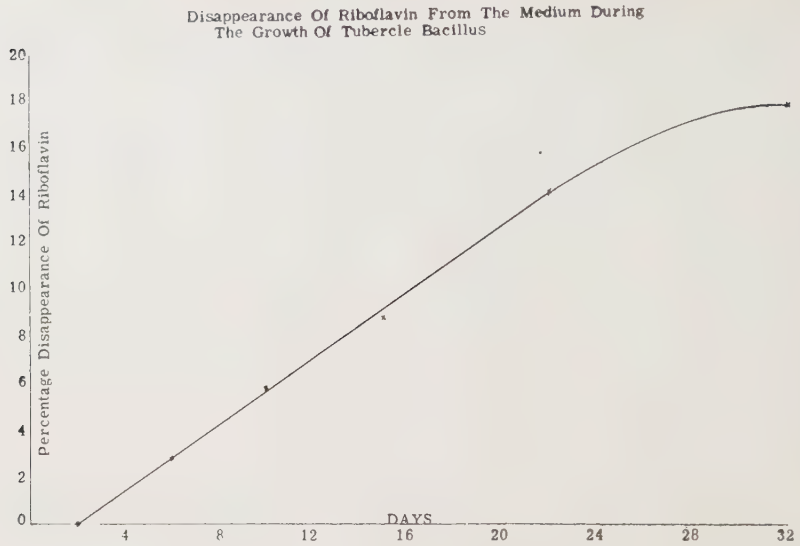

FIG. 1.

mained sterile, one-half the number of flasks were kept as controls while the remaining were inoculated with tubercle bacillus. All flasks were incubated at 37°C. At intervals of 3 days to one week 5 ml samples were removed from each flask and autoclaved at 15 lb for 10 minutes. Smears were made at each sampling to exclude any contaminated organism. If present the sample was discarded. The samples were subsequently centrifuged at high speed for 10 minutes to remove all solid particles and/or bacteria. The supernatant fluid was used for the analysis of riboflavin content, which was determined microbiologically according to a modified method of Snell and Strong[8] and fluorometrically by a composite procedure of several methods.[8]

Results and Discussion. Effect of riboflavin on growth of tubercle bacilli. The results of the study are summarized in Table I. Each figure represents the average of 10 to 20 different sets of readings. With the medium free from riboflavin the growth of tubercle bacilli was similar to that in media containing different levels of riboflavin, namely, 0.25, 0.5 and 1.0%. The addition of vitamin A, thiamine and biotin, individually or together, to the medium with or without riboflavin also did not influence the growth of the organism.

Effect of growth of tubercle bacillus on the level of riboflavin in the medium. The results of this study are shown in Fig. 1. During the first 22 days of incubation the percentage of disappearance of riboflavin from the medium plotted against time in days gave a straight line. After that time the curve began to flatten off. The disappearance of riboflavin apparently was proportional to the growth of the organisms. By the fourth week the growth had reached its maximum and the disappearance of riboflavin became correspondingly slower. On the whole the loss of riboflavin, however, was not as great as one would expect with the abundance of growth. It is conceivable that some dead organisms might have contributed some riboflavin to the medium. Foster[9] recently reported that riboflavin could be converted into lumichrome by an organism which he named *Pseudomonas riboflavinus nov. sp.* The lumichrome crystals were needle-like in appearance and could be isolated and identified by a characteristic absorption spectrum. In the present experiment similar crystals were first observed in stained smears and then in unstained liquid smears. Using Foster's technic

[8] Association of Vitamin Chemists, Inc., Interscience Publishers, Inc., New York, 1947.

[9] Foster, J. W., *J. Bact.*, 1944, **47**, 27.

of isolation it was possible to obtain purified crystals showing similar characteristics as those reported by Foster. It appears therefore that during the growth of tubercle bacillus riboflavin may be converted into lumichrome. This would furnish an explanation, at least partly, for the high incidence of riboflavin deficiency among tuberculous patients.

The finding that addition or increased concentration of riboflavin in the medium did not influence the growth of tubercle bacillus would lead us to believe that there is no danger of promoting the growth of the organism by therapeutic administration of riboflavin.

Summary. A slightly virulent strain of human tubercle bacillus was cultured in a vitamin free medium or in media to which riboflavin with or without other vitamins and biotin were added.

It was found that the presence or absence of riboflavin made no difference in the growth of the tubercle bacillus. On the other hand during the growth of the organism there was found a slow but steady disappearance of riboflavin from the medium. The presence of lumichrome crystals in the media containing riboflavin after growth of the tubercle bacillus indicated that the loss of the vitamin was due to conversion to lumichrome.

The writer is indebted to Dr. D. W. Watson and Mr. R. J. Heckly of the Department of Agricultural Bacteriology for criticism and assistance in the execution of the bacteriological technics, to Dr. C. A. Elvehjem, Department of Biochemistry, for his advice and encouragement in this work, and to Messrs. M. Moinuddin and C. C. Clayton for assistance in the riboflavin assays.

17002

Recovery of Psittacosis Virus from Chicks Hatched from Inoculated Eggs.

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Investigations[1] of psittacosis infections in psittacine and other birds have shown that active infection with the virus (*Miyagawanella psittaci*) is encountered in young birds, even nestlings. This has suggested that infection is transmitted from latently infected adults either directly to the nestling or congenitally through the egg.[1] Furthermore the virus has been reported to have been recovered from ovaries and egg yolk in the oviduct of parakeets.[1] The possibility exists therefore, that congenital transmission may be important in dissemination of the virus among psittacine birds, pigeons, and other susceptible species.

In this laboratory an effort was made to secure experimental evidence concerning transmission of *M. psittaci* by the egg in avian species. Isolations of virus were attempted from chicks hatched from eggs which had been inoculated during various stages of embryonic development.

Although psittacosis infection in chickens is not known to be widespread this species has been found naturally infected[2] and the ready accessibility of chicken eggs and familiarity with technics for handling the infection in them made this species a logical choice for preliminary work.

The strain of virus used, No. 4, had been isolated in this laboratory from a psittacine bird (*Aratinga* sp.) and maintained by serial egg passage for at least 14 transfers. It was approximately 10^4 times more lethal for white mice by intracranial inoculation than by the intraperitoneal route and was well adapted to growth in allantoic cavity and yolk sac of the developing chick embryo.

1 Meyer, K. F., *Medicine*, 1942, **21**, 175.

2 Meyer, K. F., and Eddie, A., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 522.

TABLE I.
Recovery of *Miyagawanella psittaci* from Chicks Hatched from Eggs Inoculated after Various Periods of Incubation.

Days, incubation	No. eggs inoculated in yolk sac	No. eggs inoculated in allantoic cavity	No. chicks hatched	Recovery of virus from chicks by age in days*					
				0-1	2-9	10-19	20-29	30-39	Total
0	83		14	0/1	0/5				0/6
4	56		12	0/4	0/8				0/12
7	53	32	12	0/4	0/2				0/6
8	126	48	8	1/8†					1/8
9	67		12	0/4	0/5	0/2			0/11
10	65		35	0/10	0/9	0/7	0/8		0/34
11	31		16	0/4	0/5	0/3			0/12
12	57		14	0/5	0/2	0/2			0/9
14	202		88	14/20	8/20	2/13	1/15	0/10	25/78
15	109		82	4/15	1/15	2/17	0/10	0/13	7/70
16	58		39	0/2	3/7	1/10	0/18		4/37
18		31	26	1/5	0/5	0/3	0/13		1/26
Total	907	111	358	20/85	12/83	5/57	1/64	0/23	38/304 (12.5%)

* No. chicks infected/No. chicks examined.
† Infected chick hatched from egg inoculated in allantoic cavity.

The eggs were held at 38°C throughout the entire time of incubation. After hatching the chicks were kept in a brooder for at least one week.

After being incubated for various periods from 0 to 18 days the developing embryos were inoculated into the yolk sac or allantoic cavity with .25 ml of a dilution of virus in physiological saline found by previous titration to kill approximately one half the embryos. Nearly all chicks which hatched were autopsied and virus isolations attempted at various time intervals from 1 to 39 days after hatching. The spleen, liver, and kidney of each chick were ground together in a mortar, suspended in physiological saline and injected intracranially into 5 mice. In some instances these organs were suspended and inoculated separately. The microscopic demonstration of typical clusters of elementary bodies in impression smears taken from the brains of mice dying from 3 to 7 days after inoculation and stained by the Machiavello method was taken as evidence of the presence of *M. psittaci* in the chicks.

The table presents data concerning 1018 eggs inoculated in the yolk sac or allantoic cavity after various periods of incubation. Three hundred fifty-eight chicks were hatched. Of these 304 were examined and the presence of *M. psittaci* was demonstrated in 38 of them (12.5%). The table also shows the number of chicks infected with virus in relation to the number examined for various ages in days. A few more than half of the isolations were from chicks autopsied within 24 hours after hatching; some of these chicks were moribund. The proportion of infected chicks to those examined decreased with the advancing age of the chick. The oldest chick from which virus was isolated was 22 days old, and the egg from which it had hatched had been inoculated on the 14th day of incubation.

With the exception of the moribund recently hatched chicks, all appeared in good condition and the only abnormality noted at autopsy was an enlarged spleen in a few instances which was not correlated with the recovery of virus. Virus was recovered from separate suspensions of the kidney, spleen, or liver of 7 and 14 day old chicks. Sixteen embryos dying from the 18th to 21st day of development which had been inoculated on the 8th day were examined and virus was recovered from the internal organs of 9 of them.

Summary. The data here presented indicates that chicks will hatch from eggs which have been infected with *M. psittaci* during the course of embryonic development and will survive in apparently good condition while harboring the virus in the organs for at least

22 days after hatching. While these experiments do not furnish evidence for the congenital transmission of the virus in the chicken, it is possible that in a more susceptible species the virus could be carried more effectively through the developmental and hatching period after either experimental or congenital infection.

17003 P

Studies of the "Thrombin" Effect of Fresh Serum.*

Ralph F. Jacox and Robert P. Bays. (Introduced by W. S. McCann.)

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A factor capable of producing prothrombin conversion has been described in a previous report.[1] This factor can be easily demonstrated after thromboplastin is added to serum which contains no thrombin and only traces of prothrombin. The resultant mixture of serum and thromboplastin causes rapid coagulation of a 0.01 M oxalated plasma.† Suitable control studies revealed that this factor (designated "prothrombin-converting factor"), is not thrombin, but a substance which requires prothrombin to mediate a coagulation effect on fibrinogen. Furthermore, the coagulation of 0.01 M oxalated plasma by the serum-thromboplastin mixture could not be explained as a separate action of either component.

A study has been made of the coagulation effect of *fresh serum alone* on 0.01 M oxalated plasma. It has been assumed by other workers,[2,3] that the residual clotting action of fresh serum is related to thrombin. After conversion of fibrinogen to fibrin, thrombin was believed to combine with albumin to form inactive metathrombin,[2] or that thrombin was destroyed by an enzyme.[3] Our results reveal that the residual coagulating action of fresh serum is almost entirely due to "prothrombin-converting factor". This data confirms and extends the observations of Bordet and Gengou[4] who showed that serum coagulated whole oxalated plasma much more rapidly than it did oxalated plasma from which prothrombin was removed by adsorption with tricalcium phosphate.

The coagulation effect of fresh serum was studied by the following methods. Blood was withdrawn from human donors and placed in glass tubes with internal dimensions of 1.0 x 10.0 cm. Ordinary care was used in collecting the specimens to avoid contamination with tissue thromboplastic substances. Within 15 minutes of collection, the tubes containing the blood were placed in a 26°C water bath. Approximately 50 minutes later the serum was separated from the clot. One-tenth cubic centimeter of serum (kept at 26°C) was pipetted into 0.1 cc of a 0.01 M oxalated plasma and the coagulation time determined.

It was observed that serum, which coagulated plasma rapidly when tested within 70 minutes of the time of collection from the donor, was incapable of coagulating a buffered‡ fibrinogen solution (200-300 mg %

* Research was carried out under a grant for the study of Rheumatic Fever made by the Masonic Foundation for Medical Research and Human Welfare.

1 Jacox, Ralph F., to be published in *J. Clin. Invest.*

† All plasma used in these experiments was prepared by adding 9.0 cc of whole blood to 1.0 cc of 0.1 M potassium oxalate solution.

2 Quick, A. J., *Am. J. Physiol.*, 1938, **123**, 712.

3 Glazko, A. J., and Ferguson, J. H., *J. Gen. Physiol.*, 1940, **24**, 169.

4 Bordet, J., and Gengou, O., *Ann. Inst. Pasteur*, 1904, **18**, 98.

‡ Collidine buffer (pH 7.3) was used in all experiments.[5]

FIG. 1.

Human serum was added to 0.01 M oxalated plasma at designated intervals (abscissa). The coagulation time of serum and plasma is recorded on the ordinate. Normal serum is depicted by unbroken lines; serum from patients receiving dicumarol by broken lines. C^1 serum (obtained from a patient receiving dicumarol with prothrombin less than 20 per cent of normal) had the most prolonged coagulation effect for oxalated plasma.

concentration). The fibrinogen solution§ and the plasma were equally reactive to thrombin since each coagulated with the same speed when bovine thrombin (Upjohn Co.) was added. Fig. 1 demonstrates the coagulating effect of serum when it is added to oxalated plasma. Immediately after separation of serum from the clot, coagulation of oxalated plasma was prompt (12-40 sec.). The coagulation effect of serum from normal individuals rapidly disappeared until no clotting activity remained 100-120 minutes after the time of venipuncture. Serum obtained from patients receiving dicumarol (Fig. 1—broken line) had a significantly decreased rate of degradation of the coagulating substance. As the plasma prothrombin decreased, the "prothrombin-converting factor" rate of degradation was proportionately decreased. These results are in accord with a previous observation[1] that thromboplastin activation of serum from patients receiving dicumarol, produced a slowly decaying action of the freed "prothrombin-converting factor".

Since the degradation rate of "prothrombin-converting factor" in sera of dicumarolized subjects proceeds slowly, it has been possible to obtain sera which had little loss of coagulation effect for 0.01 M oxalated plasma, 30-60 minutes from the time of separation from clotted whole blood. Such a serum was prepared by withdrawing blood from rabbits who had been given large amounts of dicumarol 24-28 hours before collection of the serum. This serum was utilized to produce the results shown in Fig. 2. The active, slowly degrading rabbit serum was added to 0.01 M oxalated

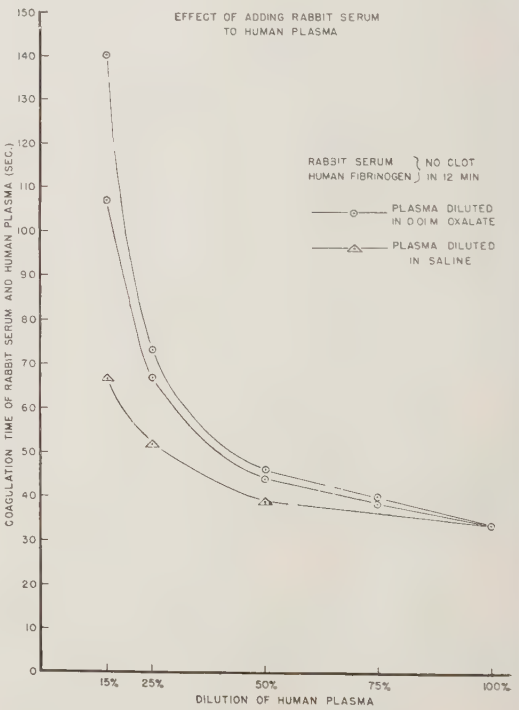

FIG. 2.

Serum obtained from a dicumarolized rabbit was added to varying dilutions of two normal human plasmas (upper 2 curves). The coagulating effect of rabbit serum is dependent upon prothrombin (fibrinogen did not clot in 12 minutes). The lower curve represents dilution of one of the two plasmas in saline rather than 0.01 M potassium oxalate, following which rabbit serum was added as described.

[1] Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 33.

§ Fibrinogen was supplied through the generosity of Armour and Company.

human plasma and to dilutions of plasma made in 0.01 M oxalated 0.9% sodium chloride solution. The coagulation time for each dilution of plasma was then determined after addition of active rabbit serum. It will be observed (Fig. 2) that the activity of the "prothrombin-converting factor" in rabbit serum is dependent upon prothrombin concentration of the plasma. A curve can therefore be constructed (Fig. 2) which resembles the reaction curve obtained in the one stage prothrobin test of Quick.[6] A similar curve can be obtained by substitution of rabbit for human plasma. This suggests that rabbit and human plasma contain nearly identical amounts of prothrombin.

It will be observed furthermore (Fig. 2), that plasma diluted in 0.9% sodium chloride solution rather than 0.01 M oxalated sodium chloride solution, produced faster coagulation in the diluted fractions when the active serum was added. This suggests that the "prothrombin-converting factor" may be partially inhibited by the oxalate ion (calcium effect?) or that "prothrombin-converting factor" may be auto-catalytically activated from plasma.

Discussion. The residual coagulating power of serum, after complete coagulation of whole blood has taken place, is not related to thrombin but rather to a "prothrombin converting factor". It is assumed that this factor is initially activated through the action of plasma thromboplastin and platelets. The "prothrombin-converting factor" then causes thrombin production by reacting with prothrombin. The thrombin quickly disappears after fibrin is formed, whereas the "prothrombin-converting factor" can be easily measured in the serum for at least 100 minutes from the time of venipuncture. In sera of patients who receive dicumarol, the "prothrombin-converting factor" effect is greatly prolonged over that observed in normal serum.

An accurate analysis of plasma prothrombin concentration can be made by use of a relatively stable "prothrombin-converting factor" obtained from rabbits receiving dicumarol. This technic of assay reproduced results obtained with the one stage determination of prothrombin by Quick's method.[6]

Owren,[7] who described factor VI (which we believe to be the same as "prothrombin-converting factor"), has reasonably credited Bordet and Gengou[4] with the first demonstration of this forgotten concept in blood coagulation.

[6] Quick, A. J., *The Hemorrhagic Diseases and Physiology of Hemostasis* (Thomas, 1942).

[7] Owren, P., *Acta Med. Scand.*, 1947, Supp. 194.

17004

Evaluation of Dubos' Solid Medium Containing Penicillin in the Isolation of Tubercle Bacilli.

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(Introduced by A. L. Bloomfield.)

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Following the observation by Dubos and Davis that a liquid medium containing Tween 80 would allow rapid, submerged growth of mammalian tubercle bacilli,[1] the diagnostic potentialities of this medium have been explored in many laboratories.[2,3] The results have in general been favorable, but two disadvantages have become apparent. First, no

[1] Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

[2] Foley, G. E., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 298.

[3] Foley, G. E., *J. Lab. and Clin. Med.*, 1947, **32**, 842.

satisfactory agent has been found to restrain contaminating organisms which are not destroyed by treatment with alkali. Penicillin is not suitable for this purpose, since in the presence of Tween it causes inhibition of tubercle bacilli in concentrations greater than 1 or 2 units per cc.[4] The other disadvantage is the necessity of staining the organisms growing in the medium to be certain they are acid-fast bacilli. If only a few tubercle bacilli are present, and particularly when there are contaminants, it is often tedious and difficult to demonstrate their presence.

More recently Dubos and his associates have described a solid medium containing oleic acid, albumin, and agar, which is relatively clear and transparent, and supports the growth of small numbers of tubercle bacilli.[5] In contrast to the liquid Tween medium, penicillin can be added in concentrations of 100 units per cc without inhibiting the growth of tubercle bacilli.[4] Virulent tubercle bacilli grow on this medium in a characteristic cord-like manner, easily observed under the low power of the microscope, which distinguishes them from avirulent tubercle and from the colonial forms of other bacteria.[6]

Because of these apparent advantages, a study was undertaken to compare the solid oleic acid-albumin medium with a standard egg-potato medium in the isolation of tubercle bacilli from clinical specimens, and to determine the effectiveness of penicillin in restraining contaminants.

Methods and Materials. Each specimen was digested with an equal volume of 4% NaOH for one hour at 37°C, with a 10 minute period of agitation in a shaking machine. Smears were made of the neutralized, centrifuged sediment, which was suspended in 0.5 cc of saline, and 2 loopfuls of the suspensions were inoculated on each medium.

The media employed were: (1) solid oleic acid-albumin, (2) solid oleic acid-albumin containing 100 units per cc of penicillin, (3) a widely used egg-potato medium containing 0.02% malachite green.[7] The media were allowed to solidify in one ounce prescription bottles, or small (1.5 by 5 cm) Petri dishes sealed with scotch tape. Only one sample of each medium was inoculated from any one specimen.

TABLE I.
General Comparison of Positive Cultures Obtained on Three Media.

	No. of cultures positive	Avg. No. days required to obtain positive culture
Total No. of specimens positive by all methods	156	
Oleic acid-albumin medium	91	14
Oleic acid-albumin medium plus penicillin	134	14.6
Egg-potato medium	120	24.4

TABLE II.
Analysis of Results from the Standpoint of Positive and Negative Cultures Obtained with Each Medium.

	No. of cultures
Positive oleic acid plus penicillin Negative plain oleic acid	55
Positive plain oleic acid Negative oleic acid plus penicillin	8
Positive oleic acid plus penicillin Negative egg-potato	20
Positive egg-potato Negative oleic acid plus penicillin	14

Results. Tubercle bacilli grew on one or more of the media in 156 of 300 specimens cultured. Of the 156 positive specimens, 61 were sputa, 91 urines, 2 gastric washings, and 2 were pus from abscesses. Comparative results are presented in Table I. The most striking observation, evident from the second column, is that positive cultures were obtained 10 days sooner, on the average, with oleic acid-albumin medium than with the egg-potato. There was no evidence that growth was delayed by the addition of penicillin to the Dubos medium.

[4] Kirby, W. M. M., and Dubos, R. J., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 120.

[5] Dubos, R. J., and Middlebrook, G., *Am. Rev. Tuberc.*, 1947, **56**, 334.

[6] Middlebrook, G., Dubos, R. J., and Pierce, C., *J. Exp. Med.*, 1947, **86**, 175.

[7] American Trudeau Society, *Am. Rev. Tuberc.* (Abstracts), 1946, **54**, Nos. 4-5.

From the standpoint of total positive cultures, the solid oleic acid-albumin medium containing penicillin appeared to be slightly superior to the egg-potato, there being 134 positives with the former, and 120 with the latter. This slight superiority is also apparent in Table II, where it can be seen that the Dubos medium containing penicillin was positive in 20 instances when the egg-potato was negative, whereas the reverse situation, (positive egg-potato, negative Dubos plus penicillin) occurred with 14 specimens.

The Dubos medium was much more efficient with the addition of penicillin than without. In 32 instances in which recovery of tubercle bacilli was prevented by the overgrowth of contaminants on the plain Dubos medium, the contaminants were suppressed on the penicillin-containing medium, and positive cultures were obtained. In one of the 8 instances in which the plain Dubos plate was positive, while that containing penicillin was negative, the tubercle bacillus was found to be inhibited by a concentration of only 10 units per cc of penicillin.

Comment. The present study indicates that Dubos' solid oleic acid-albumin medium, containing 100 units per cc of penicillin, is slightly more sensitive than a standard egg-potato medium in detecting tubercle bacilli in specimens obtained from clinical sources, and has the advantage of giving positive results 10 days earlier, on the average.

Another desirable feature is that the cord-like growth of virulent tubercle bacilli is highly characteristic on the transparent oleic acid-albumin plates; the colonies can be recognized at a glance by focusing on the agar surface with the low power of the microscope. This ability to differentiate virulent from avirulent tubercle bacilli on simple morphological grounds will probably obviate the necessity of inoculating guinea pigs; to our knowledge, no avirulent strains have been discovered which produce typical cord-like colonies.

Penicillin appears to be a highly satisfactory agent for inhibiting the growth of contaminants on the oleic acid-albumin medium. It should be noted that tubercle bacilli isolated from the urine in one patient were inhibited by 10 units per cc of penicillin; in the 134 other instances growth of the tubercle bacilli was in no way affected by a concentration of 100 units per cc of penicillin.

The widespread notion that Dubos media provide isolation from clinical specimens in 10 days, as opposed to 4 to 8 weeks with egg-potato media, is incorrect. Actually, cultures usually become positive on Dubos media from 7 to 14 days earlier than on egg-potato, regardless of the length of time required for growth to appear. With small inocula, several weeks are required; with larger inocula, growth is often visible in 7 to 14 days.

Summary and Conclusions. Of 300 clinical specimens, 134 were positive for tubercle bacilli on Dubos' solid oleic acid-albumin medium containing 100 units per cc of penicillin, and 120 were positive on a standard egg-potato medium. Without the presence of penicillin to restrain contaminants, only 91 cultures were positive on the Dubos medium. The Dubos medium was also considered superior because of the ease with which growth could be observed on the transparent agar, and because of the characteristic cord-like appearance of virulent tubercle bacilli, which apparently distinguishes them from avirulent forms, and from other organisms.

17005

Influence of Intestinal Bacteria on Synthesis of Nicotinic Acid from Tryptophan.

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Several investigators have presented suggestive but not conclusive evidence that intestinal bacteria play an important role in synthesizing nicotinic acid, making this vitamin available to the host. This subject has been reviewed elsewhere.[1]

Since it has been shown that tryptophan can substitute for nicotinic acid in the diet of most species,[2-7] and since tryptophan acts by increasing the synthesis of nicotinic acid,[8-16] it has been a natural assumption that the synthesis of nicotinic acid from tryptophan may involve the intestinal bacteria.

Ellinger and Abdel Kader[14] have reported that succinylsulfathiazole greatly reduced the urinary output of N′-methylnicotinamide when tryptophan was administered. From this and other evidence they concluded that the intestinal bacteria were involved in the conversion of tryptophan to nicotinic acid. However, Spector[12] in a somewhat similar type of experiment was led to the opposite conclusion. Junqueira and Schweigert[17] found that succinylsulfathiazole would not interfere with the conversion of tryptophan to nicotinic acid under certain conditions but would under others.

Dann and Handler[18] and Levy and Young[19] have shown that the bacteriologically sterile chick embryo can synthesize nicotinic acid. Schweigert *et al.*[20] have shown that tryptophan will increase the nicotinic acid content of the chick embryo. These experiments demonstrate that at least under certain conditions, nicotinic acid can be synthesized by the tissues independent of bacterial action.

It is the purpose of this communication to present evidence demonstrating that synthesis of nicotinic acid from tryptophan is not dependent on the intestinal bacteria in the rat.

Experimental methods. Male rats weighing approximately 250 g were maintained on a purified "nicotinic acid free" diet consisting of 12% casein, 81% sucrose, 3% corn oil, 4% minerals and the usual vitamins except nicotinic acid as described elsewhere.[10]

After at least one week on this diet, 24 hour urine specimens were collected from each rat and the basal output of N′-methly-

1 Ellinger, J., *J. Am. Med. Assn.*, 1946, **130**, 668.

2 Krehl, W. A., Teply, L. J., Sarma, P. S., and Elvehjem, C. A., *Science*, 1945, **101**, 489.

3 Briggs, G. M., *J. Biol. Chem.*, 1945, **161**, 749.

4 Wooley, J. G., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 315.

5 Singal, S. A., Sydenstricker, V. P., and Littlejohn, Julia M., *J. Biol. Chem.*, 1948, **176**, 1051.

6 Schweigert, B. S., Pearson, P. B., and Wilkening, M. C., *Arch. Biochem.*, 1947, **12**, 139.

7 Lucke, R. W., McMillen, W. N., Thorp, F., Jr., and Tull, C., *J. Nutr.*, 1947, **33**, 251.

8 Singal, S. A., Sydenstricker, V. P., and Littlejohn, J. M., *J. Biol. Chem.*, 1946, **166**, 573; *ibid.*, 1947, **171**, 203.

9 Rosen, F., Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, 1946, **163**, 343.

10 Hundley, J. M., *J. Nutr.*, 1947, **34**, 253.

11 Schweigert, B. S., and Pearson, P. B., *J. Biol. Chem.*, 1947, **168**, 555.

12 Spector, H., *J. Biol. Chem.*, 1948, **173**, 659.

13 Sarrett, H. P., and Goldsmith, G. A., *J. Biol. Chem.*, 1947, **167**, 293.

14 Ellinger, P., and Abdel Kader, M. M., *Nature*, 1947, **160**, 675.

15 Perlzweig, W. A., Rosen, F., Levitas, N., and Robinson, J., *J. Biol. Chem.*, 1947, **167**, 511.

16 Heidelberger, C., Abraham, E. P., and Lepkovsky, S., *J. Biol. Chem.*, 1948, **176**, 1461.

17 Junqueira, P. B., and Schweigert, B. S., *J. Biol. Chem.*, 1948, **175**, 535.

18 Dann, W. J., and Handler, P., *J. Biol. Chem.*, 1941, **140**, 935.

19 Levy, M., and Young, N. F., *J. Biol. Chem.*, 1948, **176**, 185.

20 Schweigert, B. S., German, H. L., and Garber, M. J., *J. Biol. Chem.*, 1948, **174**, 383.

nicotinamide determined. Each rat was then given subcutaneously 100 mg 1 (−) tryptophan dissolved in 10 cc of 0.85% sodium chloride solution and the urinary N′-methylnicotinamide output in the following 24 hours determined. All solutions were sterilized by boiling.

The animals were then matched in control and experimental groups according to their weight and to the amount of N′-methylnicotinamide excreted in response to tryptophan. The control animals were subjected to the same surgical and other procedures as the experimental animals, except that they received only saline subcutaneously while the experimental animals received the same amount of saline containing the tryptophan. A rest period of at least one week was allowed between the test dose of tryptophan and the operative procedures.

In some of the animals the entire intestine, excluding the stomach, was removed. In the remainder, only the stomach was excised. Clean but not aseptic technic was observed. The bowel stumps were simply ligated. No effort was made to anastomose esophagus and duodenum in the gastrectomized rats. Intraperitoneal sodium pentobarbital (4.5 mg/100 g) was used for anesthesia.

Immediately following operation, each rat was given either saline or saline and tryptophan subcutaneously in the same amounts as used in the control period and the urinary N′-methylnicotinamide excretion in the following 24 hours determined. Neither water nor food was allowed during this period. At the end of the urine collection all animals were carefully autopsied. N′-methylnicotinamide was determined using the method of Huff and Perlzweig.[21]

Results. The mortality rates were rather high in all the operated animals. It was found early that the rats having their intestines removed but with the distally ligated stomach remaining, would universally develop a gastric ulcer, usually with perforation and peritonitis. 3 cc of aluminum hydroxide gel placed in the stomach by stomach tube immediately after operation, prevented the complication completely and was consequently used in all rats reported here, except of course, the gastrectomised group. Only rats surviving the 24 hour post-operative period in good condition and showing no evidence of peritonitis or hemorrhage were used.

The results in the various groups are summarized in Table I. On the basal diet alone the rats excreted 120 γ of N′-methylnicotinamide per 100 g of rat per day. 100 mg 1(—) tryptophan caused a 10-fold increase in the same unoperated rats.

The operative procedure itself caused some increase in the basal excretion level, but the magnitude of the tryptophan response was so great that this was of no consequence.

In the group having all their intestine removed, the N′-methylnicotinamide output with saline alone was 246 γ per 100 g of rat per day; with tryptophan there was a 9-fold increase, the absolute response being considerably in excess of the response to tryptophan in the control period. This, we believe, demonstrates that the intestinal bacteria are not necessary for the synthesis of nicotinic acid from tryptophan.

Several investigators[22-24] have advanced evidence that the stomach is involved in some way in the nicotinic acid deficiency state. Accordingly, it seemed appropriate to determine whether the stomach might be necessary in converting tryptophan to nicotinic acid.

Gastrectomized rats (Table I) receiving saline only, excreted 182 γ of N′-methylnicotinamide per 100 g of rat per day while those receiving tryptophan showed a 10-fold increase, indicating that the stomach was not necessary for this process.

All of the rats, both in the control and operated periods, showed wide fluctuations in their N′-methylnicotinamide output as can be seen from the ranges listed in Table I. However, the output level seemed to be rather

[21] Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, 1947, **167**, 157.

[22] Sydenstricker, V. P., Armstrong, E. S., Derrick, C. J., and Kemp, P. J., *Am. J. Med. Sci.*, 1936, **192**, 1.

[23] Petri, S., Norgaard, F., Trautner, K., and Klaer, W., *Acta Med. Scand.*, 1944, **117**, 90.

[24] Gillman, T., and Gillman, J. J., *J. Am. Med. Assn.*, 1945, **129**, 12.

TABLE I.
Influence of Excision of Stomach and Intestine on Synthesis of Nicotinic Acid from Tryptophan.

No. of rats	Wt Avg	Wt Range g	Operative procedure	Treatment	N′-methylnicotinamide output μg/100 g of rat/24 hr Avg	N′-methylnicotinamide output μg/100 g of rat/24 hr Range
26	243	170-312	None	Basal diet only	120	20-413
25	244	170-312	"	100 mg L (—) tryptophan in 10 cc saline	1274	154-2821
6	257	231-312	Intestine removed	10 cc saline	246	41-512
8	247	218-310	" "	100 mg L (—) tryptophan in 10 cc saline	2156	457-4840
6	244	217-289	Stomach removed	10 cc saline	182	22-568
6	223	170-285	" "	100 mg L (—) tryptophan in 10 cc saline	1864	682-2976

characteristic for each rat, *i.e.*, if its output was low during the control period; it would also be low in the operated period and vice versa. This correlation also held when comparing the response to tryptophan in control and operated periods. Every rat* showed a significant increase in N′-methylnicotinamide output in response to tryptophan, and the response after operation was quantitatively similar to that before operation in each individual rat.

Discussion. While the experiments reported here seem to show clearly that intestinal bacteria are not required for the synthesis of nicotinic acid from tryptophan, they cannot be interpreted to mean that under normal conditions these bacteria may not produce some of the host's supply of this vitamin. It is well known that many of the intestinal bacteria can and do synthesize nicotinic acid. It remains to be proven, however, that any of this nicotinic acid is absorbed and used by the host. The experiments of Ellinger and co-workers[1] suggest that this may occur but some of their findings have not been confirmed by others.[25]

Conclusion. Rats deprived of their intestinal bacteria showed no impairment in their ability to convert tryptophan to N′-methylnicotinamide indicating that the synthesis of nicotinic acid from tryptophan does not occur in the gastrointestinal tract.

Since this paper was prepared for publication Henderson and Hankes (PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 26) have reported a study of the conversion of tryptophan to nicotinic acid using a technic similar to that described here. The results of these two studies are in essential agreement.

* We have observed an occasional rat in other experiments which will methylate neither nicotinic acid nor the nicotinic acid formed from tryptophan.

[25] Najjar, V. A., Holt, L. E., Jr., Johns, G. A., Mediary, G. C., and Fleischman, G., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 371.

17006

Inhibitory Effects of Pteroyl Glutamic Acid Preparations.

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Following the suggestion of Ross *et al.*[1] that pteroyl glutamic acid (PGA)† might exert an inhibitory effect on the glutamic acid metabolism of brain, experiments have been performed on the effect of PGA on the respiration of brain cell suspensions *in vitro*. The experiments were originally planned to test the hypothesis that PGA may act as a metabolic competitor of glutamate or other related substrates. At first, glutamate and PGA were used in approximately equimolar concentrations, 0.005 molar. Later, PGA effects were also studied in concentrations of 0.001 molar and 0.025 molar, keeping substrate concentrations 0.005 molar.

It is not to be inferred that such high concentrations of PGA bear any relation whatever to therapeutic concentrations, but only to the information that might be gained regarding their competitive action with glutamate and other substrates.

It may be noted in this connection that the glutamate concentration in human plasma is normally about 1 mg per 100 cc,[2] whereas the PGA concentration has been reported by Denko *et al.*[3] to be only 2 μg per 100 cc. On the basis of these data, the molar concentration of PGA in plasma is about 1/1000 that of glutamate.

As will appear below, inhibiting effects when present seem to be nonspecific and by no means restricted to the inhibition of respiration when glutamate is the added substrate. Furthermore, pure pteroyl glutamic acid seems to be without inhibitory effects even at a concentration of 0.025 molar.

Methods. The oxygen consumption of cell suspensions prepared from the brains of Wistar strain white rats have been measured in the Warburg apparatus at 37°C. These were prepared in a loose fitting homogenizer of the type devised by Potter and Elvehjem[4] that was driven at 100-200 r.p.m. Eighty mg fresh weight of tissue were pipetted into each flask. The gas phase was air and the shaking rate was 105 strokes per minute with a stroke displacement of 3 cm. The suspending medium was NaCl 0.136 M, KCl 0.004 M, $MgCl_2$ 0.0005 M, and phosphate buffer 0.0075 M (pH 7.4). Calcium was deliberately omitted from the incubating medium to avoid the danger of combination with or precipitation of PGA. Its omission was found to have no demonstrable effect on the respiration results.

The substrates used were: glutamate, pyruvate, lactate, succinate, and α-ketoglutarate, each at a concentration of 0.005 molar. Through the kindness of Dr. Y. SubbaRow and his associates at the Lederle Laboratories, 5 preparations of commercial PGA, a preparation of freshly recrystallized $(PGA)_R$, and a sample of the photofission product, 2-amino-4-hydroxy-6-formylpteridine, or pteridine aldehyde, were made available to us for testing. In addition, a sample of PGA was recrystallized in our laboratory.

The solutions of substrates and of PGA were adjusted to pH 7.2-7.5 before use. The elapsed time between the sacrifice of the rat and the first readings of the manometers varied from 20-30 minutes. Manometer readings were taken at 10-15 minute intervals for

* Fellow of the American Cancer Society under the administration of the Committee on Growth of the National Research Council.

1 Ross, J. F., Belding, H., and Paegel, B. L., *Blood*, 1948, **3**, 68.

† The abbreviation PGA will be used to denote pteroyl glutamic acid preparations kindly supplied us by Lederle Laboratories. $(PGA)_R$ will be used to denote freshly recrystallized PGA.

2 Bessman, S. P., Magnes, J., Schwerin, P., and Waelsch, H., *J. Biol. Chem.*, 1948, **175**, 817.

3 Denko, C. W., Grundy, W. E., and Porter, J. W., *Arch. Biochem.*, 1947, **13**, 481.

4 Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, **114**, 495.

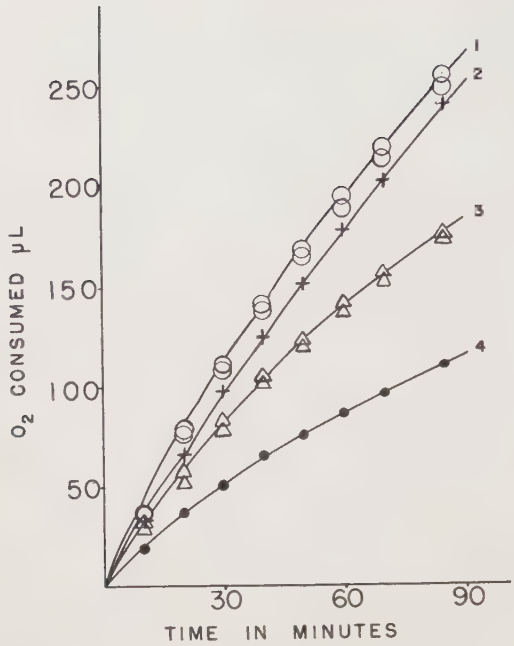

FIG. 1.

Experiment No. 50, comparison of the effect of PGA and recrystallized PGA, $(PGA)_R$, on the oxygen uptake of rat brain suspension in the presence of pyruvate. 1. Pyruvate 0.005 M with $(PGA)_R$ 0.025 M. 2. Pyruvate 0.005 M. 3. Pyruvate 0.005 M with PGA 0.025 M. 4. No substrate added. Paired circles, curve 1, and paired triangles, curve 3, denote duplicate determinations.

90 minutes and converted to microliters (µL) of oxygen consumed. Results are reported in terms of Qo_2 (µL per mg dry tissue per hour) for the 60-90 minute portion of each experiment. This period was chosen in order to increase the opportunity for any influence of added PGA preparations to become evident.

Results and Comment. The results of a typical experiment are shown in Fig. 1. This illustrates the doubling of the respiration rate produced by the presence of 0.005 molar pyruvate (compare curves 2 and 4); the absence of inhibitory effects by recrystallized pteroyl glutamate $(PGA)_R$, 0.025 molar, and the considerable inhibitory effect produced by a commercial preparation of PGA, 0.025 molar.

In Table I are presented the control Qo_2 values observed in the presence of various substrates, and the degree of inhibition produced by the addition of commercial PGA preparations in concentrations of 0.001 M, 0.005 M, and 0.025 M, respectively. It will be noted that some inhibition was produced in the presence of all substrates, or, indeed, in the absence of any added substrate, when the PGA preparation was in a concentration of 0.025 molar. The greatest inhibition was observed with pyruvate, lactate, and succinate; the least with α-ketoglutarate, glutamate, or no added substrate. No significant inhibition was observed when PGA was in a concentration of 0.001 molar; at PGA concentrations of 0.005 molar, the respiration with pyruvate as substrate was decreased 30%; with other substrates, it was not significantly altered.

In view of the absence of inhibitory effects of PGA in a concentration of 0.001 molar and in view of the non-specific nature of the inhibition encountered at PGA concentrations of 0.025 molar, it was concluded that no specific competition of PGA with glutamate was indicated by the present experiments.

Since the respiration with pyruvate seemed to be the most sensitive to additions of PGA, this substrate was used in subsequent experiments to determine (a) whether freshly recrystallized $(PGA)_R$ exerted any inhibitory effect and (b) whether breakdown products of PGA could account for the inhibition. The results of these experiments are presented in Table II.

It was found that freshly recrystallized $(PGA)_R$ exerted no significant inhibitory effect even at the high concentration of 0.025 molar, whereas experiments with commercial PGA run at the same time showed between 40 and 50 percent inhibition of respiration. From this it may be concluded that the inhibition of respiration encountered in the previous experiments was not due to the pteroyl glutamic acid present. This is in agreement with the results obtained by Franklin *et al.*[5]

Efforts were then directed to a study of the substances producing the inhibitory effect.

[5] Franklin, A. L., Regan, M., Lewis, D., Stokstad, E. L. R., and Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 523.

[6] Kalckar, H. M., and Klenow, H., *J. Biol. Chem.*, 1948, **172**, 349; Kalckar, H. M., Kjeldgaard, N. V., and Klenow, H., *J. Biol. Chem.*, 1948, **174**, 771.

TABLE I.

Effect of PGA Preparations on Oxygen Consumption of Rat Brain Suspensions. $Qo_2 = \mu L\ O_2$ consumed per hour per mg dry tissue for the 60-90 minute period of observation. Concentration of all substrates = 0.005 M.

Substrate	Control Qo_2	% inhibition PGA .001 M	PGA .005 M	PGA .025 M
Pyruvate	7.9	9	30	53
Lactate	4.7	—	—	45
Succinate	4.4	0	11	41
α-Ketoglutarate	3.8	5	13	21
Glutamate	3.8	3	16	21
None	2.3	—	—	26

TABLE II.

Comparison of the Effect of $(PGA)_R$, PGA and Pteridine Aldehyde on the Qo_2 of Rat Brain Suspensions for the 60-90 Minute Period. Substrate = 0.005 molar pyruvate. All experiments represent averages of duplicate or triplicate determinations. The concentrations of $(PGA)_R$ and PGA were 0.025 M; the concentration of pteridine aldehyde was 0.00026 M.

Exp. No.	Control Qo_2	% inhibition $(PGA)_R$, 0.025 M	PGA, 0.025 M
50	8.1	4	40
55	8.1	13	52
		Pteridine aldehyde	PGA, 0.025 M
61	8.8	32	—
62	7.4	38	59
63	7.7	27	47

Kalckar *et al.*[6] have found that the aldehyde photofission product of PGA, 2-amino-4-hydroxy-6-formylpteridine or pteridine aldehyde, is toxic to xanthine oxidase, xanthopterin oxidase, and quinine oxidase; and Lowry[7] has found that this substance is toxic to pteridine oxidase. Experiments were, therefore, carried out using pteridine aldehyde in a saturated solution. (This compound is only slightly soluble at pH 7.4, and in our experiments had a concentration of 5 mg per 100 cc or 0.00026 molar.) The results of these experiments are also presented in Table II. It was found that with pyruvate as substrate, the pteridine aldehyde in very low concentration inhibited the oxygen consumption of rat brain suspensions about 30%. Parallel experiments using an uncrystallized preparation of PGA caused an inhibition of about 50%. It would appear from these observations that, since the pteridine aldehyde is a breakdown product of PGA, it may well account in large measure for the inhibitory effects observed with commercial PGA preparations. Whether there are also additional inhibitory substances present must await further work.

Summary. 1. Freshly recrystallized pteroyl glutamic acid $(PGA)_R$ even in very high concentrations exerts no significant inhibitory effect on the metabolism of brain cell suspensions.

2. PGA preparations, not freshly recrystallized, have a marked inhibitory action in high concentrations (25 millimolar). In lower concentrations (1 millimolar), the inhibition is negligible.

3. The inhibitory action with pyruvate as substrate can be largely accounted for by the photofission product of PGA (2-amino-4-hydroxy-6-formyl-pteridine).

7 Lowry, O. H., personal communication.

17007 P

Serologic Relationship Between Streptococcus Group H and *Streptococcus sanguis.*

Rena L. Dodd. (Introduced by Grace M. Sickles.)

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Definition of streptococcus group H by Hare[1] was based on cultures from apparently normal throats. Little has since been published on strains of this serologic group. Lancefield[2] included it in her review of hemolytic streptococci. Hehre and Neill,[3] studying dextran formation by streptococci from cases of subacute bacterial endocarditis, observed that many strains producing dextran belonged to group H. Loewe and others[4] described a streptococcus isolated from cases of subacute bacterial endocarditis that required large doses of penicillin for successful treatment. The strains produced green discoloration of blood agar and dextran from sucrose. They were designated *Streptococcus* s.b.e. and, later, *Streptococcus sanguis.*[5] Their relationship to group H was not determined.

Thirty-six streptococcus strains isolated from samples of air* and 4 from cases of subacute bacterial endocarditis which were identified as group H have been studied in comparison with 6 strains of group H identified by Dr. Ronald Hare, and with 2 strains of *Streptococcus sanguis,* types I and II from Dr. James Sherman.

All strains produced small colonies on blood agar and grew well in beef infusion broth.[6] Many strains produced green discoloration of blood agar. A few, including the group H strains from Doctor Hare, produced hemolytic and green colonies. All strains fermented sucrose. The colonies of all except 8 strains from air samples and 2 from subacute bacterial endocarditis were altered in appearance when grown on blood agar containing 5% sucrose.[3] Precipitation with 1.2% alcohol of the sucrose broth cultures of 27 of the strains producing such colonies suggested dextran production. No correlation was noted between dextran production and fermentation of raffinose and salicin.[3,4]

Antisera were produced in rabbits by immunization with heated and formalin treated cells of streptococcus group H No. 36251 and *Streptococcus sanguis* types I and II. Untreated hydrochloric acid extracts[7] and extracts partially purified by precipitation with three volumes of 95% alcohol[8] were used. Reproducible results were obtained with the alcohol precipitated extracts, while some variation was noted in the reactivity of the untreated ones. Extracts of the 6 representative group H strains and of the 2 strains of *Streptococcus sanguis* reacted with group H and *Streptococcus sanguis* antisera in the precipitation test. Tests with absorbed sera demonstrated antigenic differences.

Extracts of strains from air samples and from subacute bacterial endocarditis were tested with the unabsorbed sera and 3 absorbed sera (Table I). These absorbed antisera were chosen because they served to separate the group H and *Streptococcus sanguis* strains into 4 divisions represented by

1 Hare, Ronald, *J. Path. and Bact.,* 1935, **41**, 499.

2 Lancefield, R. C., In Harvey Society, New York. Harvey lectures, 1940-41, Series 36, 251.

3 Hehre, E. J., and Neill, J. M., *J. Exp. Med.,* 1946, **83**, 147.

4 Loewe, Leo, and others, *J. Am. Med. Assn.,* 1946, **130**, 257.

5 White, J. C., Streptococci from subacute bacterial endocarditis. Ithaca, N. Y., Cornell University, 1944, 47 p. Thesis.

* These cultures were isolated from air samples taken in schools in New York State in the course of a study of the effect of ultraviolet irradiation.

6 Wadsworth, A. B., Standard Methods of the Division of Laboratories and Research of the New York State Department of Health; 3rd ed. Baltimore, Williams and Wilkins, 1947, p. 186.

7 Lancefield R. C., *J. Exp. Med.,* 1928, **47**, 91.

8 Lancefield, R. C., *J. Exp. Med.,* 1934, **59**, 441.

TABLE I.
Serologic Relationship of Representative Strains of Streptococcus Group H and *Streptococcus sanguis.*

Culture extracts		Antisera: Group H 36251				Antisera: Sanguis type I 4647				Antisera: Sanguis type II 4648			
			Absorbed with				Absorbed with				Absorbed with		
Original designation	Group and strain	Not abs.	4647	4648	36658	Not abs.	36251	4648	36658	Not abs.	36251	4647	36658
Perrier	H36251	+*	+	+	+*	+*	—	—*	—*	+*	—	+	—
Sanguis I	4647	+	—	—	—	+	+	+	+	+	—	—	—
" II	4648	+	+	—	—	+	+	—	—	+	—	+	—
Challis	H36658	+	+	—	—	+	+	+	—	+	—	+	—

+ Indicates ring formation in 10 min. and a definite cloud or precipitate in 2 hr at 35°C.
* These sera were used in the differentiation of strains.

TABLE II.
Serologic Differences Among Strains Identified as Streptococcus Group H.

Source	Colony on 5% sucrose blood agar	Number of strains with reactivity similar to: No. 36251 group H	No. 4647 *Strep. sanguis* type I	No. 4648 *Strep. sanguis* type II	No. 36658 group H
Air sample	Altered	9	4	15	0
	Unchanged	2	1	5	0
Subacute bacterial endocarditis	Altered	0	0	2	0
	Unchanged	0	0	2	0

strains Nos. 36251, 4647, 4648 and 36658.

Table II indicates the distribution among these 4 divisions of strains from air and subacute bacterial endocarditis. Of 40 streptococcus strains from samples of air and from cases of subacute bacterial endocarditis identified as streptococcus group H, eleven reacted similarly to group H No. 36251; 5 appeared similar to *Streptococcus sanguis* type I and 22 to type II, under the conditions of the test. Extracts of 2 air sample strains, one of which produced colonies with altered appearance on 5% sucrose blood agar, reacted only in unabsorbed group H No. 36251 antiserum.

Summary. Representative strains of streptococcus group H and *Streptococcus sanguis* and of 40 strains from samples of air and from cases of subacute bacterial endocarditis identified as group H were tested with unabsorbed and selected absorbed antisera produced against streptococcus group H No. 36251 and *Streptococcus sanguis* types I and II. The results of the tests indicated that the strains belonged in at least 5 antigenically different types.

17008 P

Acute Disseminated Encephalomyelitis Produced in Albino Mice.

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The production in monkeys of acute disseminated encephalomyelitis accompanied by demyelination following injection of brain tissue, first achieved by Rivers, Sprunt and Berry,[1] was not only confirmed by others, but similar lesions were later obtained with various preparations in rabbits and guinea pigs. The experimental disease has not hitherto been induced in mice. It would appear that if mice so convenient for experimental work could be shown to be susceptible, a step forward might be made. As the following results will show, it is believed that this has been accomplished.

Of 40 mice W-Swiss strain,[2] in 2 series of experiments, all were injected intramuscularly with 0.3 ml of a milky suspension containing 20 mg killed tubercle bacilli, 50 ml heavy liquid petrolatum and 10 g normal mouse brain in 50 ml saline solution. The tubercle bacilli were human type, pathogenic strain H37Rv,† acetone-dried and autoclaved for 15 minutes at 15 lb pressure. The normal mouse brain was obtained from a healthy stock of Rockefeller Institute or W-Swiss strains of albino mice. The materials were homogenized in a small-sized Waring blendor and mice were given 3 to 6 injections of the mixture at weekly or longer intervals.

Localized nodules were induced which were absorbed with difficulty and persisted for several weeks. The nodules contained grumous material, chiefly polymorphonuclear leucocytes and epithelioid cells but no visible microorganisms.

From 16 to 105 days after the first injection, 36 mice have thus far shown definite signs of involvement of the central nervous system. They exhibited all or some of the following patterns of behavior: ruffled fur; dyspnea; paresis; paralysis, generally of the hind limbs; generalized, coarse tremors; excitation alternating with somnolence; ataxia; tip-toe gait and hunched back. Subsidence of signs and later relapse were characteristic; quiescent periods endured from 2 to 15 or more days.

The chief histological changes in the brain and cord related to the marked mural and perivascular infiltration, observed more often in the white matter. The collections of cells were made up mostly of lymphocytes and microglial cells, some polymorphonuclear leucocytes and plasma cells, monocytes and compound granular corpuscles. The infiltrations tended to spread out perivascularly into the surrounding parenchyma. Vascular leucocytic or hyaline thrombi also occurred. The meninges were free from lesions or showed a spotty similar infiltration, especially about blood vessels. Neuronal degeneration was present in scattered areas, mostly in the cord, hind brain, basal ganglia and sometimes in the Purkinje cells. Disseminated, localized and diffuse glial infiltration, and small hemorrhages were visible. Demyelination was present, but not in all animals and generally in the parenchyma but this was not a prominent lesion in these early stages of illness.

Stock mice have not shown any of these signs, and repeated examinations of their central nervous tissues failed to show any apparent lesions. In addition, 20 W-Swiss mice were injected intramuscularly with 0.3 ml of acetone-ether, lipid extract of brain obtained from apparently normal mice. The concentration of lipid was 20 mg/ml and 2 to 8 injections of an emulsion of equal parts lipid and heavy liquid petrolatum were given at intervals of not less than one week. Of these 14 were killed from 14 to 113 days after the first injection, and their brains and spinal cords revealed no visible changes. Two ad-

* Lt.-Colonel, V.C., U. S. Army.

1 Rivers, T. M., Sprunt, D. H., and Berry, G. P., *J. Exp. Med.*, 1933, **58**, 39.

2 Webster, L. T., *J. Exp. Med.*, 1939, **70**, 87.

† The writers are indebted to Dr. G. Middlebrook for this strain.

ditional series of control tests were carried out: 1) 3 mice at the height of reaction to the induced encephalomyelitis were killed and suspensions of the brain and cord of each, in dilution of 10^{-1}, were injected intracerebrally into 10 mice. None of the 30 W-Swiss mice exhibited neurological signs. 2) The material used for inoculation was tested for the presence of an infective element transmissible to mice; none was found. It would thus appear that the induced encephalomyelitis is not caused by a transmissible agent present in the inocula or in the stock mice.

To conclude, disseminated encephalomyelitis was readily induced in W-Swiss mice by means of intramuscular injections of brain tissue combined with liquid petrolatum and killed tubercle bacilli—the latter being the constitutents of a modified "adjuvant" technic of Freund and McDermott.[3]

[3] Freund, J., and McDermott, K., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 548.

17009

Tests for Chemical Mutagens in *Drosophila* Using the Vaginal Douche Technic.*†

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In a previous note[1] data were presented demonstrating that the rate of mutation is increased in spermatozoa when the latter are exposed to methyl bis (β-chloroethyl) amine hydrochloride introduced into females of *Drosophila melanogaster* by vaginal douche. In addition, there seemed to be an increase in the number of mutations occurring in the egg chromosomes of females that were treated with the N mustard in this manner. It is the purpose of this paper to offer additional data which confirms these observations and to present data on the mutagenic activity of copper sulfate, formaldehyde, 2,4-dinitrophenol, hydrazine hydrate and trypsin, employing this technic. These results will be discussed together with those of other workers testing these chemicals in *Drosophila*.

Material and method. The Oregon-R wild type and the Muller-5 (sc^{s1} B In-S w^a sc^8) stocks of *Drosophila melanogaster* were used in these experiments. Virgin females from either one of these stocks were aged for 2 to 5 days, injected with a chemical substance, and then individually placed in vials with several males from the other stock. The males had been isolated soon after hatching. Injections were made as described previously,[1] but in the later experiments a set-up of ring-stand clamps, arranged to steady the hands during injection, was substituted for the micromanipulator. Females were observed for copulation for 2 hours after the injection. Only a single mating was permitted, after which the males were killed and the females placed individually in "creamers" or vials containing culture medium. Transfers to fresh creamers or vials were made every 2 to 3 days until no further offspring were produced. As in the earlier experiments, all the first generation females except those lost through accident were tested for lethal mutations involving the X chromosome from the Ore-R stock. Except for a period in the N mustard experiments reported here, in which the food was not enriched with brewer's yeast, all the first generation females were also tested for lethals involving the X chromosome from the Muller-5 stock. The plan of the crosses made for

* Part of this investigation was done at Columbia University, New York, and the Long Island Biological Laboratory at Cold Spring Harbor, N. Y.

† This work has been supported by a grant from the U. S. Public Health Service.

[1] Herskowitz, I. H., *Evolution*, 1947, **1**, 111.

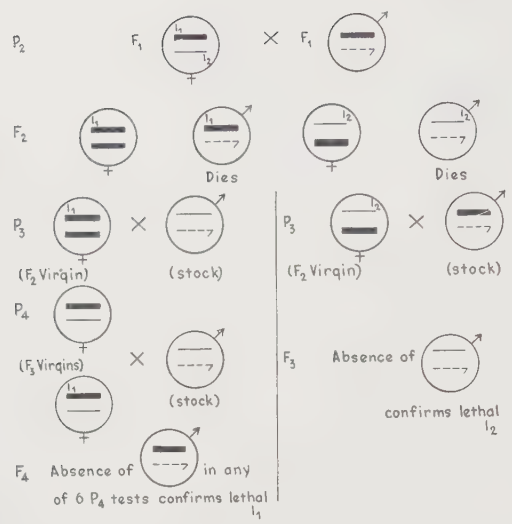

FIG. 1.

Plan of crosses to detect and confirm lethals in either X chromosome of F_1 ♀♀ from crosses between Ore-R and Muller-5 stocks. Heavy line = X chromosome from one of these stocks; light line = X chromosome from the other; broken line = Y chromosome. Single pair matings throughout.

the detection and confirmation of lethals involving either of the X chromosomes of F_1 females is presented in Fig. I where it is assumed for clarity that both X chromosomes have different lethal mutations (1_1 and 1_2). Lethal mutations originating in the sperm X chromosome (1_2) can be detected in the F_2 and retested in the F_3 generation. Lethals originating in the egg X chromosome (1_1) also can be detected in the F_2 but require an F_4 generation for confirmation.

In the N mustard experiments females failing to copulate within the 2 hour period after injection were isolated individually with one male for an unlimited period of time. The rest of the procedure was the same for these females as for those that had copulated within the time allotted, with one exception. Instead of using all the first generation females, only random samples from the first vial and from the transfer vials yielding the last female progeny were taken for tests of lethals.

F_2 cultures containing potential lethals were counted every 2 to 3 days for a month after the cross was made or until no further offspring emerged. If few or no males appeared of either the Ore-R or Muller-5 type the appropriate X chromosome was retested in a subsequent generation. Retests for mutations were performed under the same experimental conditions used for obtaining the F_2 generation. A lethal mutation was considered confirmed if the total retest population contained 1% or less of males with the tested X chromosome, and a semilethal confirmed if 1-10% of the individuals in the retest generation were males with the X chromosome tested.

Saline, prepared according to Buck and Melland,[2] was the solvent for all the chemical substances except formaldehyde, which was prepared with distilled water, and hydrazine hydrate which was used undiluted. The solutions were made just prior to use. Cultures were kept at room temperature.

Results. The fate of 414 females treated with chemicals by vaginal douche is presented in Table I. Of these, 294 females (71%) copulated within 2 hours after injection. However, this figure is composed of only 20 of the 66 Ore-R females treated (30%) as compared with 274 of the 348 Muller-5 females (79%). In a total of 359 copulations observed with Muller-5 females in these and similar experiments, 47 (13%) occurred while the females were anesthetized; among the 20 copulations observed with Ore-R females none occurred while the females were still under the effects of ether. Copulation with etherized females occurred in a variety of experiments involving the injection of 7 different chemical substances and resulted in apparently normal deliveries of sperm. The concentration of the chemical substance injected seems to affect the number of females that oviposit and, of these, the number that produce adult offspring. When the highest concentrations of copper sulfate and formaldehyde were used only 10 of 54 females that copulated (19%) deposited eggs. When intermediate concentrations of these chemicals were injected 20 of 34 females oviposited (60%), and with the lowest doses 28 of 42 (67%). With the highest concentrations of copper sulfate and formaldehyde none of the 10 ovipositing females produced adult pro-

[2] Buck, J. B., and Melland, A. M., *J. Hered.*, 1942, **33**, 173.

TABLE I.
Fate of ♀♀ Treated with Chemicals by Vaginal Douche.

Chemical	Conc., %	Date	Stock	No. ♀♀ Injected	Copulating within 2 hr	Ovipositing sterile eggs	Producing adult offspring
N mustard	4	9/48	M-5	71	55	9	26
			Ore-R	50	12	—	8
Formaldehyde	10	9/47	M-5	19	18	2	0
	5		M-5	28	22	3	13
	1		M-5	19	14	1	12
Copper sulfate	saturated sol. and 5%	9/47	M-5	46	36	7	0 (1 larvae)
	1.0		M-5	15	12	3	1
	0.2		M-5	34	28	5	9 ,,
2,4-Dinitrophenol	saturated sol.	5/47	M-5	78	53	2	32
		7/47	Ore-R	16	8	1	6
Hydrazine hydrate	85	7/47	M-5	25	23	1	16
Trypsin	conc. sol.	7/47	M-5	13	13	0	8

TABLE II.
Summary of Tests for Mutagenic Action of Several Chemical Substances.*

Chemical	Conc., %	× chromosome lethals†				
		Muller-5 ♀♀ injected		Oregon-R ♀♀ injected		
		No. (Ore-R) lethals/ No. sperm tested	No. (M-5) lethals/ No. sperm tested	No. (M-5) lethals/ No. sperm tested	No. (Ore-R) lethals/ No. eggs tested	Total lethals/ total gametes tested
N mustard	4	16/1043	6/710	7/373	3/373	32/2499
Formaldehyde	1 and 5	1/549	0/547			1/1096
Copper sulfate	0.2 and 1.0	0/183	1/150			1/333
2,4-Dinitrophenol	saturated sol.	1/751	2/739	0/562	1/562	4/2614
Hydrazine hydrate	85	1/623	2/593			3/1216
Trypsin	conc. sol.	2/506	2/506			4/1012

* Only includes tests from ♀♀ copulating within 2 hr after injection.
† All lethals were confirmed.

geny; however, when lower concentrations of these chemicals were used 35 of the 48 females that oviposited (73%) produced adult offspring.

The results of the tests for X chromosome lethals, in the daughters of females that copulated within 2 hours after injection with different chemical substances, are summarized in Table II. Only 13 lethals were observed in the 6271 sperm or egg X chromosomes tested in the formaldehyde, copper sulfate, 2,4-dinitrophenol, hydrazine hydrate and trypsin experiments. However, the N mustard treatments produced 32 lethals in 2499 sperm or egg X chromosomes tested, of which 23 lethals from 11 origins occurred in 1416 sperm chromosomes tested and 9 lethals from 8 origins occurred in 1083 egg chromosomes tested.

In the N mustard experiments females failing to copulate within the time allotted were not discarded. Instead, these females were utilized to obtain offspring that were the issue of the single pair matings taking place sometime later than 2 hours after injection. The results of egg and sperm X chromosome lethal

TABLE III.
The X Chromosome Lethal Mutation Rate in ♀♀ Copulating Later than 2 Hours After Injection with 4% N Mustard.*

Stock ♀♀ injected	No. fecund/ No. treated	No. (Ore-R) lethals/ No. sperm tested	No. (M-5) lethals/ No. eggs tested	No. (M-5) lethals/ No. sperm tested	No. (Ore-R) lethals/ No. eggs tested
Muller-5	7/16	1/444	4/363	—	—
Oregon-R	23/38	—	—	6/1091	10/1091

* All lethals were confirmed except 2 lost.

TABLE IV.
The Distribution, in the 4% N Mustard Experiments, of F_1 ♀♀ Bearing Lethals in Relation to the Total Number Tested.

Transfer vial No.	0	1	2	3	4	5	6 and 7
No. ♀♀ with sperm lethals/ No. ♀♀ tested	9/649	6/601	3/366	3/382	2/309	5/511	2/133
No. ♀♀ with egg lethals/ No. ♀♀ tested	18/649	1/564	1/188	1/268	1/246	1/489	0/133

tests with such female progeny are presented in Table III. Twenty-one lethals were obtained in a total of 2989 egg and sperm X chromosomes tested. However, only 7 of these lethals (from 6 origins) occurred in the 1535 sperm tested, whereas 14 lethals (from 13 origins) arose in the 1454 eggs tested.

Table IV shows the distribution in the various transfer vials of lethal bearing F_1 females in relation to the total number of females tested. Transfers were made every 2 days. The data come from parents copulating before as well as after the 2 hour period following injection with 4% N mustard. The 30 sperm lethals in the 2951 sperm chromosomes tested seem to be distributed at random in the transfer vials. This is not the case for the 23 egg lethals in the 2537 egg chromosomes tested. The frequency of egg lethals in F_1 females oviposited during the first two days (18/649) is significantly larger ($P < .01$) than the frequency of egg lethals in females coming from eggs deposited after that time (5/1888).

Besides the lethals, 2 visible mutants (rough and white eyes) and 2 semi-lethals occurred in the 4% N mustard experiments. These mutants occurred in the sperm X chromosomes of progeny from 3 females which had copulated within the two hours allotted. In addition, each of these parents had produced sperm lethals.

Discussion. The data in Table I offer several points of interest concerning the use and effects of vaginal douches in relation to studies of chemical mutagens in *Drosophila*. Much stronger concentrations of chemical substances may be used in vaginal douche treatments than in most other technics designed to treat gametes with chemicals for the purpose of inducing mutations. This seems to be due to the fact that only a portion of the individual is treated with the chemical substance in this procedure. Low concentrations of mutagens may be effective because of the direct contact of the chemical with the sperm.[1] However, very strong concentrations of chemical substances can produce definite harmful effects on the females treated. Failure of injected females to oviposit is due in part to the fact that many females are weakened by the chemical and die in the first few days after the treatment. This is reflected in the decrease in the percent of females which oviposit after copulation as the concentrations of copper sulfate and formaldehyde are increased. The evidence that the percent of ovipositing females pro-

ducing adult progeny is lower with the highest concentrations of copper sulfate and formaldehyde than with more moderate concentrations has several possible interpretations. High concentrations of these chemicals may cause many gametes to become non-functional and/or may produce genetic or physiological changes subsequent to fertilization that result in death before maturity. Such effects should also result in a decrease in the number of adult offspring produced when the concentration of the chemical substance injected is increased. The vaginal douche experiments that employed 0.2% and 10.0% N mustard[1] were performed under comparable experimental conditions and furnish information on this point. Unfortunately, data from the experiments with 4% N mustard are not suitable for comparison since the food used had been enriched with brewer's yeast. The average number of males and females in the F_1 progeny per fecund female was 74 in the 0.2% treatments (from 24 parents) but was only 29 (from 9 parents) in the 10.0% treatments.

In the formaldehyde, copper sulfate, 2,4-dinitrophenol, hydrazine hydrate and trypsin experiments (Table II) there were no lethals in 562 Muller-5 sperm X chromosomes tested and 7 lethals in 2535 Muller-5 egg X chromosomes tested. In the same experiments there were 5 lethals in 2612 Ore-R sperm X chromosomes tested and one lethal in 562 Ore-R egg X chromosomes tested. This suggests that the spontaneous lethal mutation rate in the Muller-5 X chromosome is much the same as in the Ore-R.[3-5] Under comparable experimental conditions we may also note the mutational response of these two chromosomes when exposed to the action of a mutagenic agent (Table II). In treatments with 4% N mustard 16 lethals occurred in 1043 Ore-R sperm tested and 7 in 373 Muller-5 sperm tested; 3 lethals occurred in 373 Ore-R eggs tested and 6 in 710 Muller-5 eggs tested. These data suggest that both the Oregon-R and the Muller-5 X chromosomes mutate with similar frequencies when exposed to N mustard. Accordingly, no distinction will be made between Ore-R and Muller-5 X chromosomes in the discussion which follows.

Vaginal douches with India ink suspensions showed that particles of the ink remained in the vagina of some virgins when these were dissected 12 hours after injection.[1] It seemed desirable, nevertheless, to test the egg and sperm lethal mutation rate in females copulating later than 2 hours after vaginal douche with 4% N mustard solution. In these experiments (Table III), there were 7 lethals in 1535 sperm chromosomes tested while in a similar number of egg chromosomes tested, 1454, there were 14 lethals. While the egg mutation rate in these experiments (14/1454) and the mutation rate in eggs from females copulating within 2 hours after injection (9/1083, Table II) seem to be comparable, the sperm mutation rate (7/1535) is considerably lower than the one in sperm deposited soon after injection (23/1416, Table II). This effect may be attributed to the removal of the mutagenic agent from the vagina sometime after the two hours allotted for copulation. The removal of the mutagenic agent may have been the result of any one or more of the following: diffusion through the body, expulsion from the vagina, chemical decay of the agent, and chemical combination with the body substances.

In the 4% N mustard treatments the lethal mutation rate in egg X chromosomes (23/2537, Tables II and III) appears to be lower than the rate in sperm X chromosomes from females copulating soon after injection (23/1416, Table II). An examination of the distribution of the egg and sperm lethals from the 4% N mustard experiments in relation to the time of hatching of the F_1 females may lead to a better understanding of the basis for the apparent difference in mutability of egg and sperm X chromosomes (Table IV). The distribution of sperm lethals in the various transfer vials seems to be at random. Accepting the hypothesis that the mutagenic agent disappears from the vagina soon after injection, this distribution is consistent with the

[3] Demerec, M., *Carnegie Inst. Wash. Yearb.*, 1946, **45**, 156.

[4] ———, *Nature*, 1947, **159**, 604.

[5] ———, *Genetics*, 1948, **33**, 337.

evidence that the genes in mature sperm are not functional.[6,7] The preponderant occurrence of egg lethals in the individuals oviposited during the first 2 days may have several explanations. Since, in all the experiments with N mustard there were no F_1 females with lethals on both X chromosomes, and in view of the data indicating that egg chromosomes mutate when sperm chromosomes do not (Table III), it is suggested that the production of egg mutations is independent of the treatment of sperm and that eggs are treated with the mutagenic agent directly. However, the means whereby eggs are exposed to the mutagenic agent still remains obscure and the apparent difference in the mutability of eggs and sperm may be the result of differences in the concentration of mutagen to which each is exposed or to a difference in the mutability of mature and immature ova.

We shall consider briefly the results of lethal tests from the N mustard, copper sulfate, formaldehyde, and 2,4-dinitrophenol experiments (Table II) with reference to the studies of other workers employing these and related chemicals in *Drosophila*. The pioneer work of Auerbach and Robson[8–10] clearly demonstrated that mustard gas and certain N and S mustards are chemical mutagens. Using the vapor and spray technics to treat mature sperm, they obtained up to 24% lethals with mustard gas and up to 13% with N mustard. Demerec[3,4] found the N mustard, methyl bis (β-chloroethyl) amine hydrochloride, to be mutagenic in treatments of spermatocytes and mature sperm using the aerosol technic. The author, in this and a previous paper,[1] has presented evidence that mutations in eggs and mature sperm are produced when this N mustard is used in vaginal douches. Such factors as the solvent for, and the concentration of, the chemical substance used should be taken into consideration in evaluating different technics for the chemical induction of mutations.

Magrizhikovskaja,[11] by bathing whole eggs, Law,[12] by injecting larvae and bathing dechorionated eggs, and Zamehof,[13] by feeding larvae, all obtained data indicating that copper sulfate is mutagenic. However, Demerec,[3] using aerosols to treat mature sperm and spermatocytes, and the author, using vaginal douches to treat eggs and mature sperm, obtained negative results with this chemical. Rapoport[14] and Kaplan[15] found positive mutagenic effects after feeding early developmental stages with formaldehyde but the author, using vaginal douches, obtained negative results. Thornton[16] has shown that 2,4-dinitrophenol decreases the growth rate and delays the moulting and pupation of *Drosophila* larvae. This drug is known to speed up the intracellular oxidative metabolism, principally of fat, in rats[17] and to inhibit phosphate uptake in staphylococci and yeast.[18] Assuming the effects of this chemical are similar in *Drosophila* it seemed desirable to test whether this chemical could influence the mutation rate via some metabolic effect. Vaginal douches with solutions of this chemical have not produced a detectable increase in the lethal mutation rate of sperm or egg X chromosomes.

Although negative results, in general, may be due to a variety of factors, it may be of interest to suggest some reasons for the failure of the aerosol and vaginal douche technics to produce mutations after treatments with copper sulfate and formaldehyde. If these

6 Muller, H. J., and Settles, F., *Z. f. ind. Abst.-u. Verer.*, 1927, **43**, 285.

7 Dobzhansky, Th., *Genetics*, 1930, **15**, 347.

8 Auerbach, C., and Robson, J. M., *Nature*, 1946, **157**, 302.

9 ———, *Proc. Roy. Soc. Edin.*, Sec. B, 1947, **62**, 271.

10 ———, *Proc. Roy. Soc. Edin.*, Sec. B, 1947, **62**, 284.

11 Magrzhikovskaja, K. W., *Biol. Zh.*, 1938, **7**, 635.

12 Law, L. W., *Proc. Nat. Acad. Sci.*, 1938, **24**, 546.

13 Zamenhof, S., *J. Genet.*, 1945, **47**, 69.

14 Rapoport, I. A., (Doklady), *C.R. Acad. Sci. U.R.S.S.*, 1947, **56**, 537.

15 Kaplan, W. D., *Science*, 1948, **108**, 43.

16 Thornton, D., *Growth*, 1947, **11**, 51.

17 Hall, V. E., Field, J., Sahyun, M., Cutting, W. C., and Tainter, M. L., *Am. J. Physiol.*, 1933, **106**, 432.

18 Hotchkiss, R. D., *Advances in Enzymology*, 1944, **4**, 153. Interscience Pub., Inc., New York.

chemicals do reach and affect eggs, spermatocytes and mature sperm, and some support for this has been presented, the negative results obtained with these technics may be due to the relatively short duration of the treatments or to differences in the mutability of the germ plasm at various stages of its differentiation.

Summary. Vaginal douches of 4% methyl bis (β-chloroethyl) amine hydrochloride produced 53 lethals in 5488 egg and sperm X chromosomes tested while only 13 lethals were observed in the 6271 sperm or egg X chromosomes tested after treatments with formaldehyde, copper sulfate, 2,4-dinitrophenol, hydrazine hydrate and trypsin. The practicality of the vaginal douche technic for the induction of mutations with N mustard in both sperm and eggs, suggested from previous experiments, is confirmed in that 30 lethals occurred in the 2951 sperm tested and 23 in the 2537 eggs tested. The number of sperm X chromosome lethals seems to be considerably higher in F_1 females resulting from single copulations within a two hour period from the time of injection (23 lethals in 1416 tests) than it is in females from copulations after this interval (7 lethals in 1535 tests); the mutation rate for the egg X chromosome under these conditions remains apparently unaffected (9/1083 and 14/1454, respectively). Whereas the 30 sperm lethals seem to be distributed at random among the F_1 females oviposited at various times, the frequency of egg lethals in individuals oviposited during the first two days (18/649) is statistically larger than the frequency of lethals in F_1 females coming from eggs deposited after that time (5/1888).

The use and effects of vaginal douche treatments are discussed and results of the experiments evaluated in the light of studies of other workers testing these chemicals in Drosophila.

I wish to express my sincere appreciation to Dr. Walter J. Burdette for many helpful suggestions concerning the manuscript and to Professor Th. Dobzhansky for numerous discussions and suggestions throughout the course of these investigations.

17010

Effect of Alloxan Diabetes on Fertility and Gestation in the Rat.*

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The discovery of the diabetogenic properties of alloxan[1] gave to the study of metabolism a tool by means of which many vexing problems may be investigated. One of these is the effect of diabetes on reproduction, a problem whose importance has been emphasized by White and Hunt[2] in extensive investigations on human diabetics. Reproduction in animals made diabetic with alloxan has been studied by Miller,[3] Friedgood and Miller,[4] Davis, Fugo and Lawrence[5] and Hultquist.[6] Reported below are some observations on the rat made diabetic with alloxan in which the problem of fertility, gestation and the via-

* From the thesis presented to the Graduate School of the University of Colorado by Jo Anne Sinden in partial fulfillment of the requirements for the M.S. degree.

1 Dunn, J. S., and McLetchie, N. G. B., *Lancet*, 1943, **2**, 384.

2 White, P., and Hunt, H., *J. Clin. Endocrinology*, 1943, **3**, 500.

3 Miller, H. C., *Endocrinology*, 1947, **40**, 251.

4 Friedgood, C. E., and Miller, A. A., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 61.

5 Davis, M. E., Fugo, N. W., and Lawrence, K. G., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 638.

6 Hultquist, G., *Acta Path. et Microbiol. Scand.*, 1948, **25**, 131.

TABLE I.
Reproduction Record of Untreated Diabetic Animals.

Rat No.	Blood sugar, mg per 100 ml: Before breeding	During gestation	Post-partum	Gestation period (days)	No. of offspring: Living	Dead	Avg body wt of offspring, g
18	398	366		22	8		5.2
29	354	328	422	23	9		5.3
30	460	396	468	23	8	2	4.8
48	372	236	452	23	9	1	5.7

TABLE II.
Reproduction Record of Insulin-Treated Diabetic Rats.

Rat No.	Blood sugar: Before insulin	With insulin	Daily insulin units	Gestation period (days)	No. of offspring per litter: Living	Dead	Avg body wt of offspring, g
24	549	280	7	22	7		4.93
29	422	236	9	23	10		5.50
30	468	228	9	22	6	1	4.79
56	474	186	7	23	8		4.75
62	476	137	9	24	4	1	5.07

bility of the fetus were investigated.

Experimental. Young female white rats, 90 to 120 days of age, from our own colony were used. The animals were kept in individual cages and were fed Purina Laboratory Chow. Lettuce was given once a week. Alloxan was administered intravenously via a tail vein in the amount of 55 mg per kilo of body weight. The method was essentially that of Lazarow and Palay.[7] The success of the production of diabetes was ascertained by the determination of the sugar content of blood obtained from the tip of the tail. A micro-modification of the method of Folin and Wu[8] was used for this determination.

The estrous cycle was followed by the vaginal smear method. When the animals were found to be in estrus, they were placed overnight with a young male of known fertility. On the following day, which was counted as the first day of gestation, the female was isolated. When the young were born they were counted and weighed within approximately 8 hours (maximum time). In all groups they were left with the mother so that her success in rearing the young might be observed.

Four groups of animals were studied. The first group received no treatment of any kind and served to establish the reproductive pattern of this strain of rats. Alloxan diabetes was produced in the animals of the second group, after which their ability to reproduce was studied without further treatment. The diabetic animals of the third group were given regular insulin twice daily with frequent determinations of the blood sugar until the estrous cycle returned. The time required for the resumption of estrus varied from 9 to 40 days. They were placed with the male at that time. The fourth group consisted of those animals which began pregnancy as diabetics and which demonstrated subsidence of hyperglycemia during the course of gestation.

Results. Normal Control Group. Fifteen rats were used as normal controls. The estrous cycle was 4 to 5 days in length. Fourteen had successful pregnancies, but 3 had to be placed with the male twice, 1 three times and 1 six times before they conceived. The remainder conceived at the first mating. The length of the gestation period was 22 to 23 days. The litters ranged from 4 to 13 in number and

[7] Lazarow, A., and Palay, S. L., *J. Lab. and Clin. Med.*, 1946, **31**, 1004.

[8] Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, **41**, 367.

the weight of the newborn animals (average weight of the young in each litter) varied from 4.65 to 6.6 g.

Untreated Diabetic Group. The animals in this group were treated with alloxan and the vaginal smears were followed daily thereafter. Estrus appeared at intervals of 3 to 8 days following the alloxan administration. There were 13 diabetic animals in this group of which 9 failed to reproduce. The placental sign was observed in 3 of the latter 9 rats and a vaginal plug was seen in 4 of the 9 after they were with the males. In these animals the blood sugar values ranged between 365 and 468 mg per 100 ml.

The data concerning the 4 rats which did bear litters are given in Table I. Rat 18 killed and ate her young soon after parturition. The young from rat 30 died on the fourth day after parturition. Rat 29 cared for her young, but on the 14th postpartum day it became evident that she was unable to nourish them adequately, and they were placed with a foster mother. They quickly gained weight and were successfully reared to the weaning age. Rat 48 reared all of her 9 live young to the weaning age.

Insulin Treated Diabetic Group. These animals were treated with regular insulin until a definite decrease in blood sugar had been established. With adequate treatment the estrous cycle was resumed in 5 of 7 animals even though the blood sugar values had not reached a normal level. The results of the reproduction experiment with this group are recorded in Table II. Of the 5 which resumed estrus, every one conceived and bore a viable litter. The young of rat 24 lived only 2 days. Rat 62 bore 4 live and 1 dead offspring, and the 4 live ones died within 3 days.

Rats 29 and 30 had borne litters while diabetic and untreated (Table I). Their estrous cycles were not resumed after the birth of their young until insulin treatment was begun. They then conceived and bore litters. Rat 29 bore 10 live rats which were reared to the weaning age and 6 living and 1 dead young were born to rat 30. The living young died within 3 days.

Rat 56 bore 8 living offspring which were accidentally sacrificed soon after birth.

Untreated Group with Transitory Diabetes. One group of 5 animals had only mild or transitory diabetes. Indeed, in 2 of these animals the blood sugar never did reach a level which would justifiably place them in the diabetic group. However, they were bred and produced litters, and the blood sugar decreased during the course of pregnancy to normal levels. The number of rats per litter varied from 5 to 13 and the average weight of the young varied between 4.6 and 6.1 g. The litter of one of these rats was born dead. These results seem to indicate that the administration of alloxan does not interfere with the ability to reproduce when it has been unsuccessful in the establishment of diabetes.

Discussion. The most recent report on this problem is that of Hultquist.[6] He produced diabetes both by pancreatectomy and by alloxan administration. Most of his animals were made diabetic after pregnancy was established and "only in a few instances" did he observe pregnancy in an already diabetic animal. He also observed that insulin improved the chances of the mother to bear young and that only one of many animals completed gestation without insulin treatment. The results herein reported show a higher percentage of successful pregnancies in untreated diabetic animals than his report seems to indicate, but also, marked improvement was observed when insulin was given, even though, again in agreement with Hultquist, the blood sugar was not regulated to non-diabetic values.

The experiments of Davis, Fugo and Lawrence[5] vary somewhat from the present ones. They observed a prolongation of the sex cycle from 4 to 5 days to 9 to 12 days with irregularity, in contrast to the observations reported above which showed one, or at the most, 2 estrous periods following a diabetogenic dose of alloxan, after which the cycle was not restored unless insulin was administered. Furthermore, none of their diabetic animals which were bred after becoming diabetic produced litters, in contrast to the 4 among 13 rats which accomplished successful gestation in the present experiments. The

smears were not examined for spermatozoa in our untreated diabetic rats after they had been with the male, and it is possible that some of the 9 animals which did not conceive failed of successful coitus.

When diabetic animals were treated with insulin their cycles returned and they were successfully bred and produced litters, in agreement with the findings of Davis, Fugo and Lawrence. The young either were not in good health or the mother's milk supply was inadequate as evidenced by the early death of all but one of the litters. Also confirming the findings of these authors, we observed that those animals which recovered spontaneously from hyperglycemia were able to reproduce. Alloxan, *per se,* in the doses used did not interfere with the ability of these animals to reproduce.

Miller[3] reported a successful gestation in 3 diabetic rats which received no insulin. They were only mildly diabetic as judged by the mild glycosuria. Friedgood and Miller[4] gave alloxan to pregnant rats on the fourteenth day of gestation. They determined that the blood of the fetuses was hyperglycemic at a level only slightly below that of the mother, and that the blood sugar of the offspring had returned to normal within one day. In the present experiments it was observed that the young of the 2 surviving litters from diabetic mothers had blood sugar values within the normal range. These determinations were performed after the young had been weaned. In consideration of these findings and the report of Dohan and Lukens[9] that short exposure of cats to hyperglycemia will produce diabetes, the resistance of the fetus of the rat to maternal hyperglycemia needs further investigation.

Abnormally large body weight is commonly observed in babies borne by human diabetics.[10] The young rats in this experiment, both from treated and untreated diabetic mothers did not exceed in weight those from non-diabetic mothers. In fact, the highest average weight in the litters occurred in the offspring of one of the mothers of the non-diabetic group.

The existence of diabetes in the mother, whether treated or untreated, did not affect the length of the gestation period significantly.

Summary. Rats were made diabetic with alloxan and their ability to reproduce was studied under various conditions. The following observations were made: (1) the estrous cycle ceased in diabetic females and was restored by the administration of insulin; (2) 4 of 13 untreated diabetic animals, which were bred before the estrous cycle ceased, produced litters; (3) insulin treated diabetic animals reproduced in spite of a continuing hyperglycemia; (4) transient hyperglycemia did not interfere with reproduction and (5) diabetes in the mother did not result in hyperglycemia in the offspring in the few animals tested.

[9] Dohan, F. C., and Lukens, F. W. D., *Endocrinology,* 1948, **42**, 244.

[10] Joslin, E. P., Root, H. F., White, P., Marble, A., and Bailey, C. C., The Treatment of Diabetes Mellitus, Lea and Febiger, Philadelphia, 1946, 773.

17011

Effect of Succinylsulfathiazole on the Urinary Excretion of Folic Acid by the Rabbit.*

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Previous studies[1] showed that the growing rabbit does not require a dietary source of pantothenic acid or riboflavin when a purified diet is fed. Further, the urinary and fecal excretion of these vitamins and also of biotin greatly exceeded the intake and was not appreciably reduced when 1% of succinylsulfathiazole was included in the diet. Preliminary experiments indicated that the folic acid excretion was greatly reduced when the sulfa drug was fed; consequently these studies have been extended to obtain further information on the excretion of folic acid by rabbits receiving purified diets with and without the addition of succinylsulfathiazole.

Experimental. In the first experiment New Zealand white rabbits with an initial weight of approximately 1060 g were fed the basal ration or the basal ration plus 2% succinylsulfathiazole. Six rabbits were used in each group. The basal ration was composed of corn oil 8%, cellulose 12%, Salts IV[2] 4%, vitamins A and D concentrate 0.5%, cerelose to 100% and tocopherols, 2-methyl 1-4-naphthoquinone, thiamine, riboflavin, nicotinic acid, pyridoxine, Ca pantothenate, choline, inositol, biotin and para-aminobenzoic acid in adequate amounts.[1] After 3-4 weeks on experiment quantitative urine collections were made and the amount of folic acid in the urine was determined with pteroylglutamic acid as the standard and *S. faecalis* R the test organism.[3] Individual urine collections were made for 3 day periods from the 4th to the 10th week alternately for 3 of the 6 rabbits in each group. The technics used for the urine collections and preservation were described previously.[1] Other groups fed the basal ration + 2.0 mg of pteroylglutamic acid per kg of ration and the basal ration + pteroylglutamic acid and succinylsulfathiazole were included in this experiment, however the urinary excretion of folic acid was not determined for these groups.

A second experiment was carried out with 8 rabbits fed the basal ration and 8 fed the basal ration plus succinylsulfathiazole. The initial weight of the rabbits averaged 1720 g. Urine collections were again made and the folic acid excretion determined. The results obtained for both experiments are shown in Table I.

Results and Discussion. The rabbits receiving the basal ration without added sulfa drug grew at a normal rate. The rate of gain for other groups which received 2.0 mg of pteroylglutamic acid per kg of ration was not increased. The rate of gain has been shown to increase when liver extract is added.[4] The addition of 2% succinylsulfathiazole to the ration markedly reduced the rate of gain of most rabbits, however the rabbits with a larger initial weight, particularly in the second experiment, were not severely retarded in weight gains. The rates of gain of the control groups included in the first experiment that received pteroylglutamic acid with or without the sulfa drug were normal.

* We are indebted to Dr. L. D. Wright, Sharp and Dohme, Inc., for supplying the succinylsulfathiazole and to Dr. T. H. Jukes, Lederle Laboratories Division, American Cyanamid Co., for the synthetic pteroylglutamic acid used in this study.

† Present address: Division of Biochemistry and Nutrition, American Meat Institute Foundation, University of Chicago.

1 Olcese, O., Pearson, P. B., and Schweigert, B. S., *J. Nutrition*, 1948, **35**, 577.

2 Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 459.

3 Teply, L. J., and Elvehjem, C. A., *J. Biol. Chem.*, 1945, **157**, 303.

4 Kunkel, H. O., Simpson, R. E., Pearson, P. B., Olcese, O., and Schweigert, B. S., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 122.

TABLE I.
Effect of Ingesting Succinylsulfathiazole on Urinary Excretion of Folic Acid by the Rabbit.*
(μg excreted per rabbit per day).

Dietary regimen	Weeks on experiment						
	4	5	6	7	8	9	10
Exp. I							
Basal	2.3	1.5	2.6	3.6	10.9	4.8	8.9
Basal + S.S.	1.1	0.5	1.6	1.8	2.2	1.9	2.4
Exp. II							
Basal	2.6	3.0	7.8	16.0	—	3.8	3.4
Basal + S.S.	1.0	1.0	2.5	3.7	—	0.4	0.5

* The values are averages of 3 individual 3-day collections for the first experiment and of 6 individual collections for the second experiment.

The addition of 2% of succinylsulfathiazole markedly reduced the urinary excretion of folic acid active compounds. This effect was evident by the 4th week and although the excretion of folic acid remained at a low level for the succinylsulfathiazole group, a considerable increase in the apparent synthesis of folic acid was observed for the basal group, particularly from the 7th to 10th weeks. Although accurate measurements of folic acid in the ration were not achieved due to the low level, the estimated dietary intake of 1 μg per day approximated the amount excreted by the sulfa fed group and was much lower than the amount excreted by the basal group. It may be concluded therefore that the rabbit is capable of synthesizing sufficient folic acid, as well as pantothenic acid, riboflavin, and biotin, presumably by intestinal microorganisms to meet its needs and that the apparent synthesis of folic acid can be markedly reduced by the inclusion of 2% succinylsulfathiazole in the ration.

Other tests showed that the inclusion of succinylsulfathiazole in the ration did not reduce the hemoglobin level of the blood. Rabbit blood was found to contain folic acid conjugase;[5] however the level of apparent free folic acid in blood was very low (<1.0-4.0 millimicrograms per ml) and the data obtained do not permit an accurate evaluation of the folic acid blood levels of the groups receiving and not receiving the sulfa drug.

Summary. The urinary excretion of folic acid by rabbits fed purified diets with or without the addition of succinylsulfathiazole was studied. The inclusion of the drug markedly reduced the urinary excretion of folic acid. This effect was evident from the 4th to the 10th week of the experiments.

[5] Simpson, R. E., and Schweigert, B. S., *Arch. Biochem.*, 1949, **20**, 32.

17012

Dissociation Among Lancefield's Group "B" Streptococci of Human and Bovine Origin.*

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One hundred and thirty bovine and 26 human strains of Lancefield's group "B" streptococci were studied within one month after isolation.

Primary isolations were made on beef heart infusion agar (Difco) which contained 3%

* This investigation was conducted with the cooperation of the following institutions: San Juan City Hospital, Bayamón District Hospital and the Veterans Hospital at San Patricio.

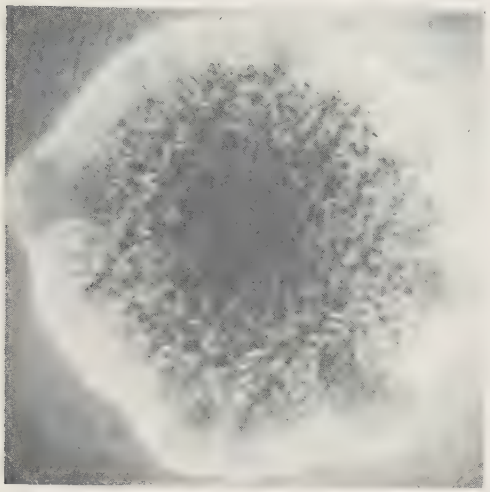

FIG. 1.
"M" colony, 60 hr, 37°C. Blood agar. × 49. Notice sectors indicating M ⟶ S variation. The granulations are most probably accumulations of red blood cells.

of rabbit or horse blood. The morphology of the colonies was studied on rabbit blood agar plates. The plates (streak) were incubated aerobically at 37°C and examined with a hand lens and a microscope. Only isolated colonies with ample free surrounding space were selected for study. In studying the colonial morphology by reflected light the plate had to be tilted at the proper angle in order not to miss certain details.

Four distinct morphological types were encountered among cultures recently isolated from milk or pus obtained from cows with clinical mastitis. A description of these follows:

1. Colonies slightly raised, comparatively large, about 2.5 mm after 48 to 72 hours incubation, edge entire or slightly wavy. Surface white and glossy by reflected light. With the low power of the microscope tiny white solid structures were seen scattered throughout the body of the colony giving to it the appearance of a tiny drop of slightly curdled milk. Consistency of thin paint. Semitransparent periphery and a rather opaque central area (Fig. 1). In the bacteria from some of these colonies definite capsules could be demonstrated either from young cultures on blood agar or in peritoneal exudate from mice. Broth cultures showed a homogeneous turbidity with a scanty powdery sediment. The cocci occurred singly, in pairs or in very short chains. Some strains were pathogenic and others were avirulent for mice. This description corresponds with the mucoid (M) colony of Dawson, Hobby and Olmstead.[1]

When colonies of this type were incubated at 37°C for 48 to 72 hours frequently wedge shaped, rather opaque outgrowths appeared at the periphery. (Fig. 1). These outgrowths when transplanted gave rise to rather opaque colonies which corresponded in all respects to the second type of colony encountered in primary cultures which we shall now describe.

2. Colonies raised, edge entire or very slightly wavy. Surface dull and usually slightly granular. Consistency of thick paint. They could easily be broken into *soft thick* membranous portions. Relatively opaque when compared with the "M" colonies. (Fig. 2). Growth in broth was homogeneous or very finely granular. The cocci occurred mainly in short chains. No capsules could be demonstrated. This description corresponds with the smooth (S) type colony of Dawson *et al.*[1]

It is apparent that the opaque wedge-like growths of the mucoid colonies represent a mucoid to smooth variation.

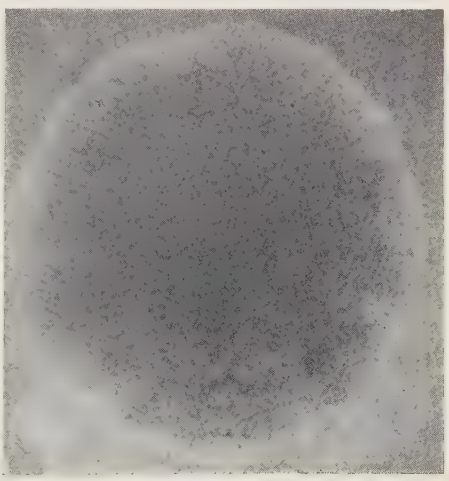

FIG. 2.
"S" colony. 48 hr at 37°C on blood agar. × 41. Granulations most probably accumulations of red blood cells.

[1] Dawson, M. H., Hobb, G. L., and Olmstead, M., *J. Infect. Dis.*, 1938, **62**, 138.

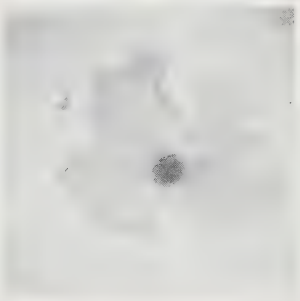

FIG. 3.
"M" colony with prominent rough outgrowths. One week on blood agar at 37°C. × 5.

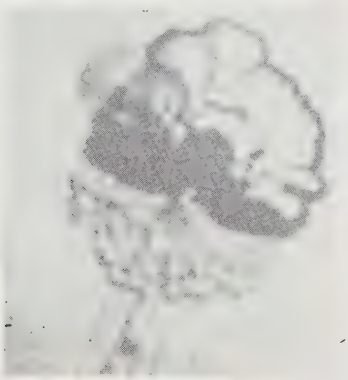

FIG. 4.
"S" colony with rough outgrowths. Four days on blood agar at 37°C. × 20.

If "S" or "M" cultures were left in the incubator from three days to one week rough, large, fan-like outgrowths usually arose from the periphery of the colonies. (Fig. 3 and 4). A large proportion of the "S" and "M" cultures produced these outgrowths. Transplants to broth from these outgrowths gave a cottony sediment with a clear supernatant as contrasted with the homogeneous turbidity obtained with "M" and "S" cultures. The cocci were larger than normal and occurred chiefly in long tangled chains. Colonies obtained from transplants of these rough sectors to 3% rabbit blood beef heart infusion agar showed a slightly raised conical central portion and a rather flat peripheral zone. Upon prolonged incubation they became flatter and flatter until their appearance was much like that exhibited by the "R" colonies obtained in primary cultures as we shall see later. This type of colony conforms in many respects with the "R" (rough) type of Dawson.[1]

3. Colonies prominently flat, composed of a thin membrane with the appearance and consistency of a spider web. Edge of colony very irregular and fimbriated. In some instances small, smooth papillae are distributed throughout the colony. (Fig. 5). Cottony sediment with clear supernatant in broth. Cocci usually larger than normal arranged in long

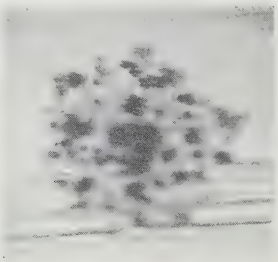

FIG. 5.
Papillated rough colony. × 15. Blood agar. Four days at 37°C.

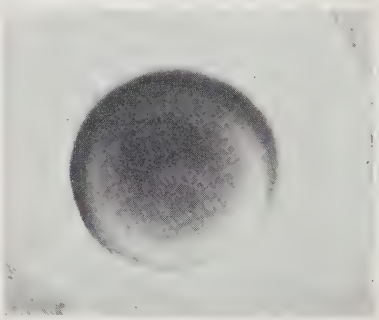

FIG. 6.
Twenty-four-hr-old precursor of smooth-ravined (SRa) colony. × 30.

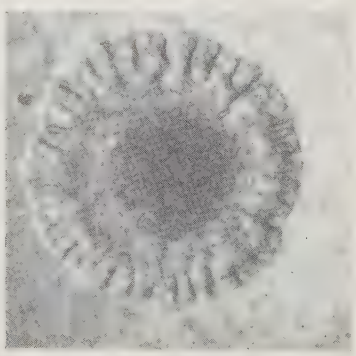

FIG. 7.
Smooth-ravined (SRa) colony. 72 hr on blood agar at 37°C. × 30.

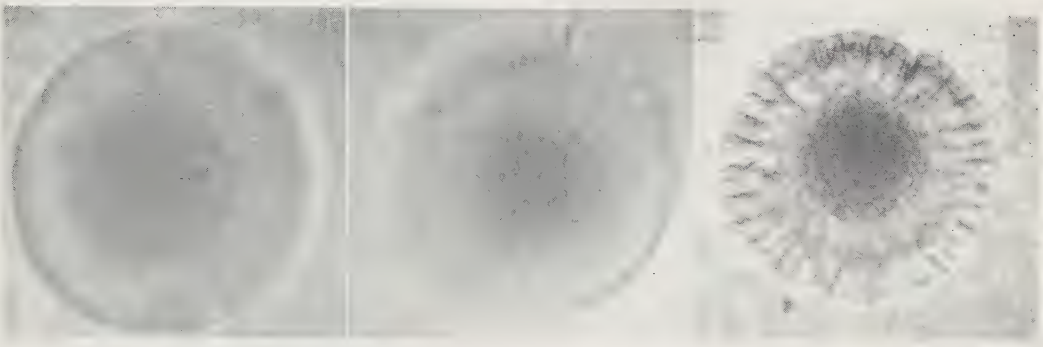

FIG. 8.
Stages in the development of the smooth-ravined (SRa) colony. Large type. Three-day-old colonies on rabbit blood agar. × 30.

tangled chains. These correspond with the rough (R) type of Dawson[1] and were very similar to the rough outgrowths already described obtained from "S" and "M" colonies.

4. Colonies were conical, with a prominently white, dense, raised central disc. The rest of the colony was much less dense. The whole colony was smooth and the surface glossy when 16 to 18 hr old and approximately 1 to 1:5 mm in diameter. (Fig. 6). A small and large type of colony were encountered. The central disc was usually of a soft consistency or exceptionally rather hard so that it could be removed with the wire without disturbing the rest of the colony which at this stage was of medium hardness and could be broken easily with an inoculating wire into thick membranous portions. Occasionally these colonies were of such consistency that they could be shifted from place to place without disturbing their characteristic morphology.

After from 48 to 72 hours incubation at 37°C the central white disc remained as described above. However, the rest of the colony showed by this time a deeply ravined glossy surface with a grayish milky appearance by reflected light. (Fig. 7). Now the colony was harder but it could be broken with the wire into thick membranous portions. The growth in broth was homogenous with scanty powdery sediment or finely granular with slightly foccular or slightly ropy sediment. The cocci occurred typically in chains of medium size. In some instances colonies in the different stages of development were encountered on the same plate (Fig. 8). So far as we know this type of colony has not been described before. We are designating it, and shall refer to it in this discussion as the "SRa" or the *smooth-ravined* colony.

If these smooth ravined cultures were left in the incubator at 37°C for 4 or 5 days some colonies showed glossy, soft pseudopod-like peripheral outgrowths. (Fig. 9). Upon prolonged continued incubation (one or two weeks) these smooth soft portions in turn gave rise occasionally to rough outgrowths similar to those already described as arising from "S" and "M" colonies.

All the morphological types described above were found in primary cultures from the mastitic udder. No rough (R) or *smooth-ravined*

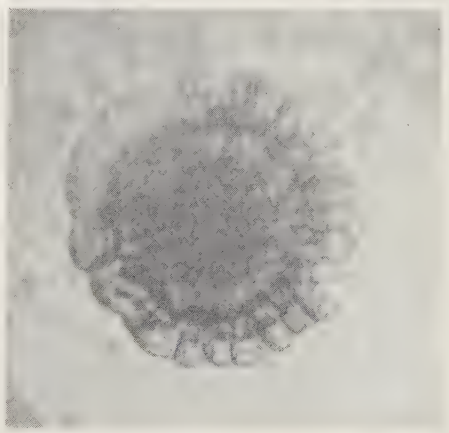

FIG. 9.
Smooth-ravined (SRa) colony with glossy outgrowth. Five days on blood agar. Large type of colony. × 30.

(SRa) types were found in primary cultures from human sources. All the human strains produced either "M" or "S" colonies. However, the S → R and M → R dissociations were produced with relative facility with human strains as described above for cultures of bovine origin.

The fermentative and other biological properties of most of these strains were tested[2] and no correlation was found between these properties and colonial morphology.

Serums prepared in rabbits with formalin treated suspensions of "M" cultures gave good precipitin reactions with formamide extracts of "M" or "SRa" organisms of human and bovine origin. Rabbit serum prepared with "S" or "SRa" suspensions reacted similarly. "M", "S" and "SRa" serums reacted negatively with formamide extracts prepared with the corresponding "R" variants.

The serums obtained from rabbits after several series of inoculations with formalin treated "R" organisms always gave negative precipitin reactions with formamide extracts of the homologous "R" suspensions or extracts prepared from the parent "M" or "S" human and bovine cultures.

These results show conclusively that the group precipitinogen extracted by Fuller's method is greatly diminished or completely lacking in the "R" phase of Lancefield's group "B" streptococci of human and bovine origin. One must keep in mind this fact in the identification of "R" variants of *Strep. agalactiae* obtained in primary cultures from the bovine udder.

Two "M" cultures which were virulent for mice (0.05 cc of 18 hr broth culture killed mice in 24 hours) gave rise to "R" variants which were completely avirulent (1 cc intraperitoneally).

All the bovine and human cultures, irrespective of their dissociative phase at the time they were tested, were resistant to streptomycin *in vitro* when tested by the filter paper disc method of Bondi *et al.*[3] All the bovine and human strains were sensitive to penicillin *in vitro* but the sensitivity varied greatly with the different strains. This variation in susceptibility to penicillin was independent of the origin and colonial morphology of the cultures.

Summary. The dissociation among 130 bovine and 26 human strains of Lancefield's group "B" streptococci has been studied. Four different morphological types of colonies (rough, smooth, mucoid and smooth-ravined) are discarded in detail. No correlation was found between colonial morphology and the biological properties and susceptibility to penicillin. All strains were resistant to streptomycin *in vitro*. Cultures from rough outgrowths of mouse virulent mucoid parent colonies were completely avirulent for mice. Formamide extracts of mucoid, smooth and smooth-ravined cultures reacted positively in precipitin tests with homologous and heterologous rabbit serums prepared with mucoid, smooth and smooth-ravined cultures and with commercial group "B" serum. Formamide extracts prepared from rough cultures always gave negative precipitin tests with good precipitating rabbit serums prepared with the corresponding mucoid, smooth and smooth-ravined parent cultures.

[2] Pomales-Lebrón, A., Morales-Otero, P., and Baralt, J., PROC. SOC. EXP. BIOL. AND MED., 1947. **61**, 410.

[3] Bondi, A., Spaulding, E. H., Smith, D. E., and Dietz, C. C., *Am. J. Med. Sc.*, 1947, **213**, 221.

17013

A Histochemical Method for Localizing Cholinesterase Activity.*

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With technical assistance of Elizabeth E. Matthews.

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The exact site of cholinesterase (ChE) activity in relation to the structural elements of various tissues has been a matter of uncertainty. Information on this subject up to the present time has been derived indirectly from studies correlating the over-all ChE activities of different samples of tissues with the relative proportions of certain cells or structures present.[1,2] Gomori[3] has published a histochemical method for the direct visualization of the location of ChE activity, in which the substrates used are long-chain fatty acid esters of choline. However, his protocols indicate that the rate of splitting of these compounds by various tissues is extremely low in comparison with that of acetylcholine (ACh); with such enzyme sources as rat brain, which is said to contain only specific ChE,[4] and purified preparations of erythrocyte and electric organ ChE, little or no hydrolysis of these compounds occurred. It seems likely, therefore, that this technic localizes only nonspecific esterases, many of which hydrolyze ACh, but not specific ChE which is considered of physiological significance in the transmission of nerve impulses. In the method reported here, the substrate employed is acetylthiocholine (AThCh) which, as shown below, is hydrolyzed at a more rapid rate than ACh by both specific ChE and nonspecific esterases, presumably because of the weaker

$$\begin{matrix} O & & O \\ \| & & \| \end{matrix}$$

linkage of the —C—S— than of the —C—O— bond. The present technic appears to indicate the location of all enzymes classified under the general term "cholinesterase", and does not permit differentiation between the several types of ACh-splitting enzymes. Such a distinction is now being attempted by the use of the sulfur homologues of acetyl-beta-methylcholine and benzoylcholine as substrates, and by means of selective inhibitors.

$$\begin{matrix} & O \\ & \| \end{matrix}$$

Acetylthiocholine Iodide ($CH_3CSCH_2CH_2N(CH_3)_3I$) was first synthesized by Renshaw *et al.*,[5] who noted that its pharmacological actions were similar to those of ACh but of briefer duration. We have employed their method of preparation with minor modifications.† Glick[6] reported that this ester was hydrolyzed more rapidly than ACh by horse serum ChE. The same relationship was found to hold for all sources of ChE tested in the present study, as shown in Table I. ChE activity was determined manometrically by a

* This work was supported by the National Institute of Health, John and Mary Markle Foundation, and the Chalfant Fund.

1 Marnay, A., and Nachmansohn, D., *J. Physiol.*, 1938, **92**, 37.

2 Sawyer, C. H., and Hollinshead, W. H., *J. Neurophysiol.*, 1945, **8**, 137.

3 Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 354.

4 Mendel, B., and Rudney, H., *Science*, 1943, **98**, 201.

5 Renshaw, R. R., Dreisbach, P. F., Ziff, M., and Green, D., *J. Am. Chem. Soc.*, 1938, **60**, 1765.

† We are grateful to Dr. W. Gump of Givaudan-Delawanna, Inc., Dr. Max Tishler of Merck & Co., and Mr. P. W. Blume of Michigan Chemical Corp., for generous supplies of the initial compound, beta-chloro-ethyl dimethyl ammonium chloride. The latter organization supplies this substance commercially. Dr. Eric W. Martin, Philadelphia, Pa., suggested modifications of the synthesis. It has been brought to our attention that AThCh can be obtained from the Bios Laboratories, Inc., New York City.

6 Glick, D., *J. Biol. Chem.*, 1939, **130**, 527.

TABLE I.
Rates of Hydrolysis of Choline Esters by Enzymes from Various Sources.

Enzyme source	Micromoles CO_2 per g or cc per hour		Ratio Q AThCh / Q ACh
	ACh	AThCh	
Rat Brain	224	367	1.64
'' Erythrocytes	35	98	2.80
'' Serum	23.4	62.3	2.67
Cat Erythrocytes	11.4	38.3	3.36
'' Adrenal	50.6	104.5	2.06
Purified bovine Erythrocyte ChE (Winthrop-Stearns) 1.0 mg	1,820	2,700	1.49

modification of Ammon's[7] method. The initial concentration of each substrate was 5×10^{-3} M, which approaches the optimal range of ACh concentration for specific ChE,[8] and the pH of the solutions after gassing with 5% CO_2–95% N_2 was approximately 8.0. Parallel determinations with both substrates were run on all enzyme sources for periods of 10 to 30 minutes, depending on the rate of CO_2 production, and corrections were made for non-enzymatic hydrolysis. The concentration of Cu++ (0.002 M) employed in the histochemical procedure was found, under the same conditions, to produce no appreciable inhibition of rat brain homogenate ChE with either substrate, whereas 10^{-3} M di-isopropyl fluorophosphate (DFP) caused complete inhibition.

Histochemical Procedure. The method consists of incubating teased preparations or frozen tissue sections in a medium containing 4×10^{-3} M AThCh and 0.002 M copper glycinate at a pH of 8.06, saturated with copper thiocholine, for 10 to 60 minutes. The saturation of the substrate solution with the reaction product (in this case copper thiocholine) was first suggested in relation to enzymatic histochemical technics by Friedenwald and Becker.[9] It has the double advantage that diffusion of reaction product is greatly diminished or suppressed, thus enhancing the crispness of the histological localization, and, in addition, since the controls are incubated in the same solution and since the controls show no deposit, of demonstrating that the localization found in the test specimens is not due to mere staining or adsorption. Following this the sections are subjected to two brief rinsings in distilled water saturated with copper thiocholine, then immersed in ammonium sulfide solution, which converts the white precipitate of copper thiocholine to a dark brown amorphous deposit of copper sulfide. They are then affixed to slides with albumen, after which they are either mounted in glycerine or dehydrated through increasing concentrations of alcohol and counterstained as desired. Controls were run by allowing freshly-cut sections to stand in 10^{-3} M DFP in 0.85% saline for 30 minutes at room temperature, following which they were washed in distilled water and treated by exactly the same procedure as described above. In all preparations studied to date, the controls were completely free of the characteristic staining seen in the untreated preparations described below.

Reagents

Solution 1: 3.75 g Glycine, 18.0 cc N KOH, distilled water q.s. 100 cc.

Solution 2: 0.1 M copper sulfate.

Solution 3: 14.5 mg AThChI, 0.75 cc H_2O, 0.25 cc solution 2. Centrifuge. Decant supernatant solution from precipitated cupric iodide.

Copper Thiocholine prepared by alkalinizing a solution of AThCh in copper glycinate to pH 12.0 with KOH, allowing to stand overnight at room temperature, collecting the precipitate and washing with water.

7 Ammon, R., *Arch. ges. Physiol.*, 1933, **233**, 486.

8 Augustinsson, K.-B., *Acta Physiol. Scandinav.*, 1948, **15**: Supplementum 15, 1.

9 Friedenwald, J. S., and Becker, B., *J. Cell. Comp. Physiol.*, 1948, **31**, 303.

FIG. 1.
Rat intercostal muscle, × 125.

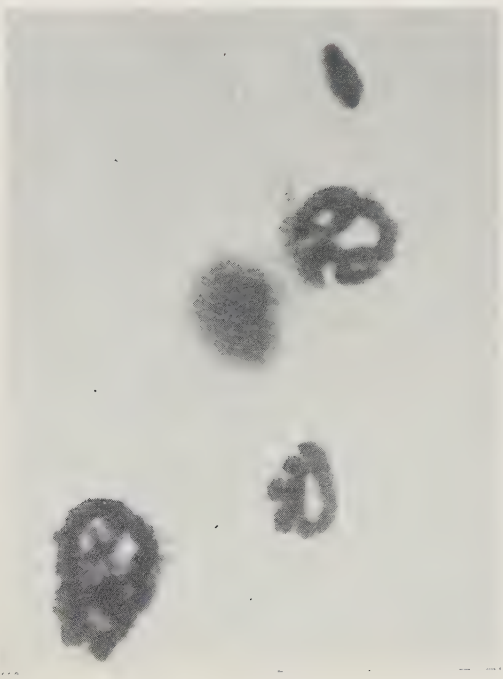

FIG. 2.
Rat intercostal muscle, × 600.

Incubation Solution. 0.4 cc solution 1, 0.2 cc solution 2, 8.6 cc H_2O; add small trace of copper thiocholine, disperse thoroughly, and place in water bath at 37° for at least 15 minutes, stirring occasionally. Immediately before using, add 0.8 cc solution 3, filter.

Results. The following descriptions and accompanying photomicrographs† are of preparations which were not counterstained. Other tissues studied to date, the interpretation of which has been less clear, include liver, spleen and heart.

Striated muscle. Teased preparations of intercostal muscle of the rat (Fig. 1, 2) following incubation for 10 minutes, revealed distinct structures identified as motor endplates. It is interesting to note that the picture bears a striking resemblance to that obtained of the same muscles of the mouse by Couteaux,[10] using the Janus green-ammonium

FIG. 3.
Rat medulla, region of gracile and cuneate nuclei, × 125.

† Photomicrographs taken by Mr. Delbert Parker.

[10] Couteaux, R., *Rev. Canadienne de Biol.*, 1947, **6**, 563.

molybdate stain which is considered selective for the subneural apparatus. Couteaux postulated that ChE is probably most concentrated in this region, a hypothesis with which the present findings are compatible. When preparations were allowed to incubate for longer periods, the sarcolemma and nuclei also showed varying degrees of staining.

Brain. Sections of rat medulla (Fig. 3,4), incubated for 40 minutes, were stained very deeply in the regions of the gracile and cuneate nuclei. At these sites large neurons and the immediate portions of their processes were revealed distinctly, as well as numerous glial nuclei which appeared to be predominately microglial. Because of the thickness (30 μ) of the sections studied with the present technic, it could not be determined whether or not the irregular background staining was due entirely to intracellular material. Elsewhere, cell bodies, processes and nuclei of fibrous astrocytes and nuclei of microglia were visible. The general architecture of the former appeared to outline various fiber tracts.

Autonomic Ganglia. Incubation of sections

FIG. 5.
Cat superior cervical ganglion, × 125.

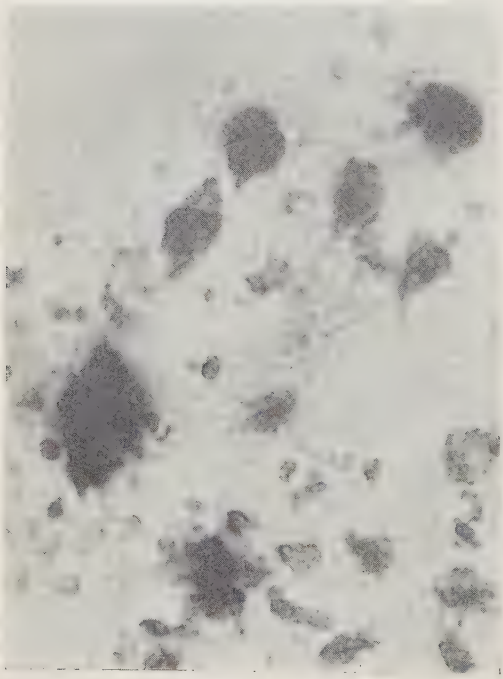

FIG. 4.
Rat medulla, region of gracile nucleus, × 600.

FIG. 6.
Cat superior cervical ganglion, previously incubated in 10^{-3} M DFP, × 125.

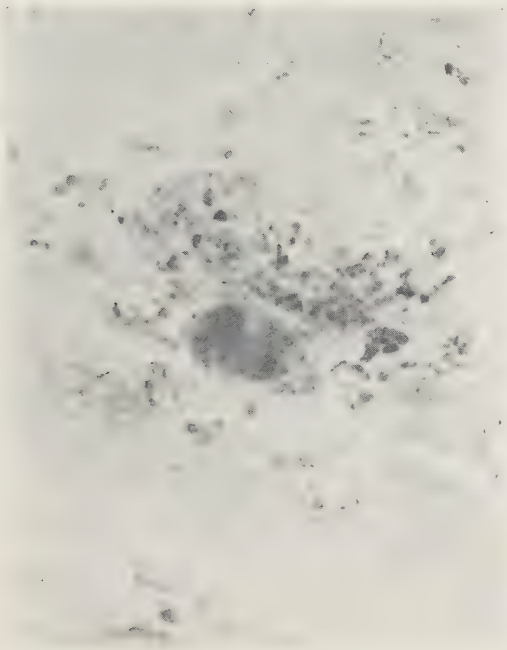

FIG. 7.
Cat superior cervical ganglion, × 600.

of cat superior cervical ganglion for one hour resulted in extremely dark staining (Fig. 5) which was completely lacking in the DFP-treated controls (Fig. 6). In the associated trunk, the nuclei of Schwann's cells were distinguishable, along with the general outline of the nerve fibers. Besides the heavily-staining ganglion cells and nuclei of satellite cells, both of which were seen better in sections incubated for one-half this period (Fig. 7), a fairly heavy background stain was present, forming an irregular pattern. No definite identification has been made of the structures represented by the discreet particles forming this background. It was much less dense in sections of the stellate ganglion of the same animal, where the ganglion cells, in consequence, stood out more sharply.

In sections of the rat ileum (Fig. 8), ganglion cells were stained in the areas of the submucous and myenteric plexi, and appeared to be far more numerous in the latter. The nuclei and general outlines of the muscle fibers were also stained.

Adrenal. The ganglion cells and nuclei of

FIG. 8.
Rat ileum, region of myenteric plexus, × 600.

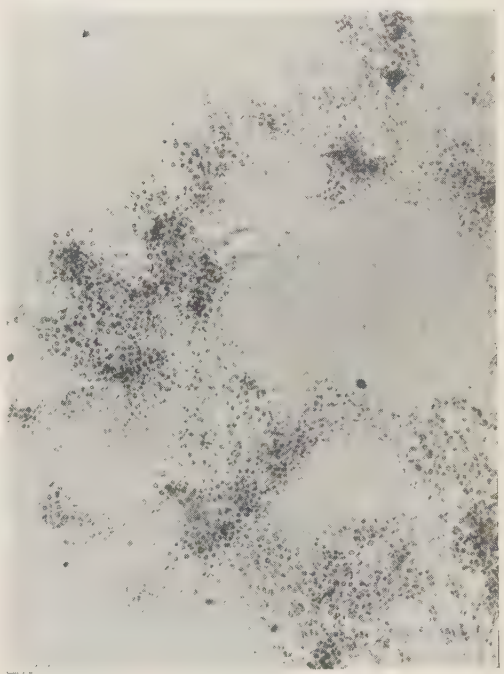

FIG. 9.
Cat adrenal medulla, × 125.

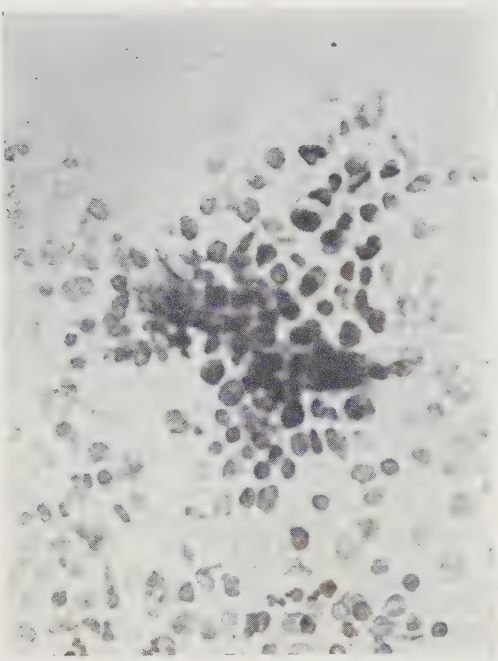

FIG. 10.
Cat adrenal medulla, × 600.

what appeared to be chromaffin cells of the adrenal medulla (Fig. 9, 10) of the cat were deeply stained, forming a pattern of irregular cords. No staining was seen in the cortex.

Comments. In any histochemical procedure the question arises as to whether or not the area where a precipitate is seen represents the actual site of enzymatic action. The alternative interpretation is that diffusion of the initial product of hydrolysis or of the subsequent reaction product has occurred, followed by a precipitation at an adjacent site. In the present study, the possibility of diffusion prior to precipitation was minimized by the previous saturation of the incubation medium with the final reaction product, copper thiocholine. The efficacy of this procedure in promoting immediate precipitation is of course dependent upon the adequate penetration of both copper thiocholine and copper glycinate from the surrounding medium to the sites of the enzyme in the tissues. That such occurred may be inferred from the fact that in preliminary tests in which saturation with copper thiocholine was omitted, precipitation occurred only after prolonged incubation (18-24 hours) and was manifested, after treatment with ammonium sulfide, by the appearance of clumps of acicular crystals of copper sulfide which bore no resemblance to cellular structures.

The intensity of the specific stain at any site is dependent upon several factors, foremost among which are probably the concentration of the enzyme and its reaction velocity or turnover number. The localization of specific ChE is favored in the present technic by a substrate concentration (4×10^{-3} M) in the optimal range for that enzyme; however, nonspecific esterases can also hydrolyze the substrate at this concentration, although at a slower rate. Methods which are being studied at present for localizing separately specific ChE and nonspecific esterases have been mentioned above.

Summary. A histochemical method is presented for localizing ChE activity by incubating tissue sections in a medium containing acetylthiocholine, copper glycinate and copper thiocholine. Results obtained with several tissues containing specific ChE are described and illustrated.

17014

Effect of Gold Administration on Liver Function in Dogs.

Martin Gunter and A. C. Ivy.

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It is a well-established fact that patients with rheumatoid arthritis experience much relief following the development of jaundice.[1] The jaundice apparently must be of the immediate direct reacting type (*i.e.* nonhemolytic), in order to be benficial. Hench[2] has suggested that the ameliorating effect of gold in rheumatoid arthritis is perhaps analogous to, if not basically identical with, that induced by intercurrent jaundice or pregnancy.

Hartung[3] found jaundice to be an uncommon sequela of gold salt therapy (2 in 800 cases). Altogether Hartung reports 4 patients who developed jaundice following a course of gold therapy. The onset of jaundice was accompanied by an improvement in the patients' subjective and objective symptoms, with relapses occurring almost immediately after the jaundice subsided. Hartung feels it to be highly questionable whether or not the jaundice was actually due to the gold salt therapy.

In this laboratory we have been concerned with the relationship of liver function to rheumatoid arthritis, and some of us[4] have described a method by means of which artificial jaundice of the direct reacting type may be produced. The present study was undertaken in order to ascertain the effect of gold administration on liver function.

Experimental. The following tests were carried out:

1. Rose Bengal Clearance[5]
2. Alkaline phosphatase[6]
3. Direct and indirect quantitative van den Bergh reaction for Bilirubin[7]

Two dogs, which were in good health and which weighed 13.2 and 14.1 kg were used.

Following the determination of control values for the above tests, 25 mg of gold in the form of sodium gold thiomalate (myochrysine) were administered to each dog intramuscularly each day for one week. Again their liver functions were tested. Next the gold administration was repeated, using 75 mg per day instead of 25. After one week liver function tests were repeated. Then, following a period of 2 weeks, the tests were again repeated.

Following this we administered 100 mg per day of gold thioglucose (solganal-B) in a 10% aqueous solution. Four days following the last administration, all the tests were repeated. The results are shown in Table I. It is apparent from the results that in no case did any of the liver functions tested, vary beyond the values accepted as normal.

Discussion. Rawls *et el.*[8] showed that patients with rheumatoid arthritis in many instances exhibit abnormal values for tests of liver function. Apparently considerable disturbance of liver function is needed before an amelioration of rheumatic symptoms is noted. Hence it appears unlikely that gold acts by producing sufficient effect on the liver to produce the relief from arthritic symptoms in this manner. On the other hand the poorly functioning liver of rheumatoid arthritis patients may be more easily open to the effect of gold therapy. In order to decide that, more quantitative studies on patients receiving gold therapy are needed.

1 Hench, P. S., *Med. Clin. No. Am.*, 1940, **24**, 1209.

2 Hench, P. S., *Ann. Int. Med.*, p. 618, April, 1947.

3 Hartung, E. F., *Med. Clin. No. Am.*, p. 553, May 1946.

4 Snapp, F. E., Gutman, M., Li, T. W., and Ivy, A. C., *J. Lab. Clin. Med.*, 1947, **32**, 321.

5 Stowe, W. P., Delprat, G. D., and Weeks, A., *Am. J. Clin. Path.*, 1933, **3**, 55.

6 Shinowara, Jones and Reinhart, *J. Biol. Chem.*, 1942, **142**, 921.

7 Hoffman, W. S., *Photelometric Clinical Chemistry*, New York, 1941, p. 231.

8 Rawls, W. B., Weiss, S., and Collins, V. L., *Ann. Int. Med.*, 1939, **12**, 1455.

TABLE I.
Effect of Gold Therapy on Liver Function.

Dog	Alkaline phosphatase, units %	Rose Bengal clearance, %	van den Bergh reaction: Direct, mg %	van den Bergh reaction: Indirect, mg %
Control Values.				
1	6.2	119	0.0	0.2
2	4.4	109	0.0	0.1
Following 1 wk of daily administration of 25 mg of gold (sodium auro thiomalate).				
1	6.4	94	0.1	0.0
2	5.8	96	0.0	0.0
Following 1 wk of daily administration of 75 mg of gold (sodium auro thiomalate).				
1	7.2	111	0.1	0.0
2	8.2	111	0.1	0.0
Two wks later. No gold given during this interval.				
1	6.3	96	0.0	0.1
2	6.4	—	0.0	0.0
Following 1 wk of daily administration of 100 mg of gold thio glucose.				
1	7.0	115	0.0	0.0
2	6.4	95	0.0	0.0

In this study on dogs, the doses used, in terms of human therapy, were large, and a course of treatment which would require weeks in man is compressed into one week.

Summary. 1. Two dogs received gold in the form of sodium auro thiomalate and of thioglucose daily for a total of 3 weeks.

2. Determinations of Rose Bengal Clearance, alkaline phosphatase, and direct and indirect quantitative van den Bergh revealed no pathological changes in liver function.

3. These observations do not support the view that the ameliorating effect of gold therapy in rheumatoid arthritis is due to the effect of the gold on the liver.

17015

Failure of Thyroidectomy and Thiouracil to Protect Rat Liver from Acute Carbon Tetrachloride Injury.

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(Introduced by K. M. Endicott.)

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Leach and Forbes[1] have reported that sulfanilamide administered to rats protected the liver from inhaled carbon tetrachloride. In a subsequent experiment Forbes, Leach and Williams[2] found that sulfanilamide also retarded the development of hepatic cirrhosis induced by CCl_4. They suggested that the protective action might be related to the inhibition of thyroid activity known to be induced by sulfanilamide. György and Goldblatt[3] and György, Rose and Goldblatt[4] have shown that thiouracil and propylthiouracil exert a marked preventive effect on the incidence and degree of dietary cirrhosis in rats. These authors also suggested that the pre-

1 Leach, B. E., and Forbes, J. C., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 361.

2 Forbes, J. C., Leach, B. E., and Williams, G. Z., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 47.

3 György, P., and Goldblatt, H., *Science*, 1945, **102**, 451.

4 György, P., Rose, C. S., and Goldblatt, H., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 67.

TABLE I.
Comparison of Liver Damage Produced by Carbon Tetrachloride in Thyroidectomized and Thiouracil-fed Rats and in the Pair-fed and *ad lib.* Controls.

Rat group	No. of rats	Extent of lesion*	Hydropic degeneration†	Necrosis‡	Fatty metamorphosis*
Thyroidectomized	13	++	+++	±	+
Pair-fed controls	13	++	+++	+	++
Ad lib. controls	11	±	+	∓	+
Thiouracil-fed	8	++	++	+	+
Pair-fed controls	8	++	++	+	+

* Extent of lesion, fatty metamorphosis—graded on a + to ++++ basis.
† Hydropic degeneration—plus values represent proportion of cells in involved areas showing this type of alteration.
‡ Necrosis —, ∓ rare cell, ± occasional cell, + a few cells.

ventive effect was mediated through the thyroid gland. Handler and Follis[5] in experiments with rats concluded "A decreased level of thyroid activity induced by thyroidectomy, thiouracil or p-aminobenzoic acid feeding prevents or retards the development of hepatic necrosis or fibrosis associated with choline and cystine deficiencies."

The experiments reported here show the failure of reduced thyroid activity in the rat to protect the liver against subcutaneously administered carbon tetrachloride.

Materials and Methods. The animals used were male rats of the Sprague-Dawley strain and weighed, at the beginning of the experiments, between 84 and 120 g. In one experiment 13 rats were thyroidectomized. An equal number of pair-fed rats served as one control group. A second control group of 11 rats were allowed diet *ad libitum*. The diet consisted of ground pellets (Purina laboratory chow) in which calcium carbonate was incorporated at a 2% level. The thyroidectomized rats were started on this diet 7 days before operation, and after operation were given 0.25 cc of 10% calcium gluconate intramuscularly for a few days, not exceeding 5 days for any rat. After 44 days all rats were injected subcutaneously with 0.05 cc CCl_4 (in equal parts of mineral oil) per 100 g of body weight, and killed and autopsied 24 hours later. The "thyroid area" in the operated rats was examined under 4X magnification for the presence of thyroid tissue; none was found.

In another experiment conducted at the same time, 8 rats were fed the ground pellet diet (without calcium carbonate) in which thiouracil was incorporated at a 0.1% level for 38 days and at a 0.2% level for another 45 days. The source of fluid for these rats was a saturated aqueous solution of thiouracil. A group of 8 pair-fed control rats received the same diet without thiouracil; water without thiouracil was the source of fluid. At the end of the 83 day period, all rats were injected with CCl_4 and killed as in the first experiment.

In each experiment the liver and thyroid gland or "thyroid area" of all rats were removed at autopsy and fixed in 10% formalin and prepared for microscopic study by paraffin embedding, and staining with azure eosinate. Frozen sections of the fixed livers were stained with oil red O.

Results. On microscopic examination the extent and type of liver cell damage were graded on a one plus to four plus basis. The liver cell injury, which was characteristically centrolobular in location, comprised loss of diffuse and granular cytoplasmic basophilia, irregular and inconstant slight cytoplasmic hyalinization, cytoplasmic oxyphilia, necrosis, hydropic degeneration and fatty metamorphosis. Of these changes only the last 3 listed, together with the extent of the lesion, were used as a basis of comparison. The results are shown in the accompanying table.

It is evident that the extent of the lesions and the degree of hydropic degeneration were not suppressed by thyroidectomy or thiouracil feeding. It is felt that the slight difference in amount of necrosis between the thyroidec-

5 Handler, P., and Follis, R. H., Jr., *J. Nutrition,* 1948, **35**, 669.

tomized rats and their pair-fed controls is within the range of error inherent in the system of grading. Thyroidectomized rats showed less fatty metamorphosis; the difference is not great but may be significant. The livers of rats fed *ad libitum* showed extremely little damage except the one plus fatty metamorphosis. The only known differences between these rats and the pair-fed controls of this experiment were the greater amount of food consumed and a faster growth rate.

The few sections made through the "thyroid area" of each operated rat failed to reveal any thyroid tissue. The thyroid gland of all thiouracil fed rats showed marked epithelial hypertrophy with severe depletion of colloid. The thyroid glands of all other rats were histologically normal.

Discussion. The inhibition of thyroid activity suggested by Forbes *et al.*[2] as a possible explanation for the liver protective effect of sulfanilamide is not supported by our findings. It should be pointed out, however, that in their experiment the CCl_4 was administered by inhalation.

György *et al.*[3] and Handler *et al.*[5] found that a decreased level of thyroid activity protected the livers of rats against parenchymal necrosis in dietary cirrhosis studies. In view of the findings in the present study it would seem that the mechanism of protection against carbon tetrachloride necrosis[2] and the necrosis occurring in dietary cirrhosis is different. In this connection Handler *et al.*[5] found that sulfasuxidine lessened the tendency of the livers of choline-deficient rats to become necrotic and scarred even though the thyroid glands of these animals were essentially normal.

Summary. The lowering of thyroid activity by (1) thyroidectomy and (2) the feeding of thiouracil in a stock diet, failed to prevent or suppress hydropic degeneration or necrosis of the rat liver induced by subcutaneous administration of carbon tetrachloride.

17016

Effect of Temperature on Transmission of Light by Cell Free Body Fluid in *Phascolosoma gouldii.*

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The biological fluids of higher animals have been studied intensively and extensively from the point of view of chemical, physical, and physiological characteristics. The physical nature of the serum of mammals, for example, has been investigated with great thoroughness by Du Noüy who showed that at 56°C "profound modifications in the structure of the proteins and of the lipoprotidic complex" become evident.[1]

On the other hand, very little is known about the physical behavior of invertebrate body fluids. In order to ascertain the relationship of invertebrate fluids to those of higher animals, a series of experiments was made to test the effect of heating on the transmission of light by the cell free body fluid in *Phascolosoma gouldii.*

Material and methods. In general the procedure was similar to that followed by Du-Noüy in studies on horse serum. Body fluid was carefully removed from 10 phascolosomas, using a 5 cc hypodermic syringe fitted with an 18 gauge needle. Approximately 1 to 3 cc of pink fluid were obtained from each worm. The pooled fluid was then centrifuged for about 10 minutes in an ordinary clinical cen-

[1] Du Noüy, L., *Studies in biophysics. The critical temperature of serum (56°).* New York, 180 pp. 1945.

trifuge. The cell free supernatant fluid was drawn off with a pipette and introduced into a soft glass test-tube about 6″ by 5/8″ in size. The open end of the tube was then quickly drawn out and sealed in a gas flame. Another tube, carefully matched with the first, was similarly prepared but was filled with distilled water. It was used as a control. Altogether 12 tubes, representing over 100 worms, were prepared.

Some of the tubes were heated for 5 minutes in a carefully controlled water bath at temperatures from 24° to 90°C. Other tubes were heated for 20 minutes at like temperatures. An electric photometer, with a 610 mμ filter, was adjusted to register 100% light transmission on the galvanometer scale, when the control tube was in place. (The light source, filter, absorption tube, and photocell were linearly arranged.) This value was recorded as incident light (I_o). Before each test the needle setting was adjusted with the control tube in place. The control tube was replaced by the tube containing the heated serum (which was cooled to room temperature) and the value on the galvanometer scale was recorded as transmitted light (I). The temperature of heating for each tube was increased after each photometric test until coagulation of the cell free fluid took place.

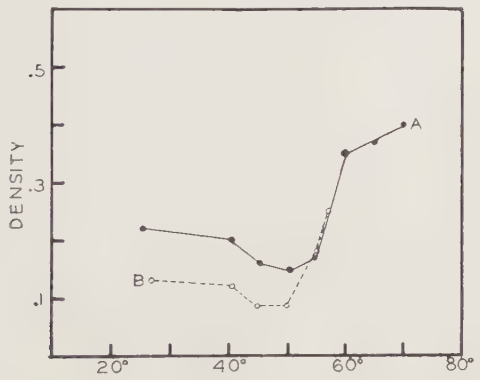

FIG. 1.

Graph obtained by plotting the optical density ($d = \log I_o/I$) against the temperature, in degrees Centigrade, at which the body fluid was heated. Curve A, fluid heated for 5 minutes; curve B, for 20 minutes.

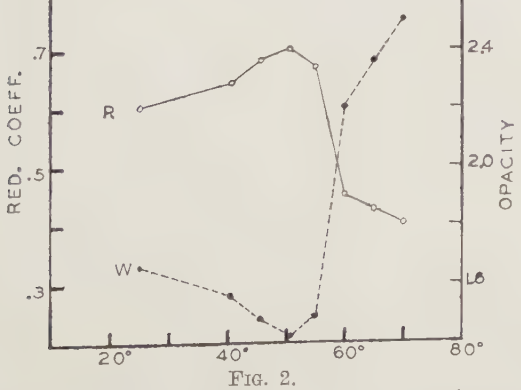

FIG. 2.

Graph showing the curves obtained by plotting the reduction coefficient ($r = I/I_o$) and the opacity ($W = 1/r = I_o/I$) against the heating temperature in degrees Centigrade. Curve R, reduction coefficient; curve W, opacity.

Results. The results are summarized in Fig. 1 and 2. Fig. 1 shows the results of plotting optical density ($d = \log I_o/I$) against temperature of heating. Curve A represents fluid heated for 5 minutes; curve B for 20 minutes. The results show that at about 40°C there is a decrease in optical density which suddenly increases rapidly beginning at about 50°C. (There is an evident increase in light transmission at about 40°C followed by a decrease beginning at 50° to 55°C).

Fig. 2 shows the results of plotting reduction coefficient ($r = I/I_o$) and opacity ($W = 1/r$) against temperature of heating. The curves are for fluid heated for 5 minutes. It is evident that at 40°C there begins an increase in reduction coefficient and a decrease in opacity. At about 50°C the reduction coefficient begins to fall, at first slowly, then rapidly and the opacity increases similarly. Curves for fluid heated for 20 minutes have the same configuration but are higher or lower on the graph.

The temperature for coagulation of the fluid varied greatly with different samples. This agrees with the results of Andrews who says that the fluid may coagulate at any temperature from 63° to 80°C.[2]

Discussion. The pooling of a series of experimental individuals to obtain directly a median value has been used for various investigations with favorable results.[3] In the

[2] Andrews, E. A., *Johns Hopkins Univ. Circ.*, 1890, **9**, 65.

[3] Greiff, D., and Pinkerton, H., *J. Exp. Med.*, 1945, **82**, 193.

present work it was essential in order to have large enough samples for measurement. The median values are quite consistent.

If it is assumed that the body fluid contains protein particles dispersed in a fluid medium, then if the particles become smaller the light transmitted shifts toward the red end of the spectrum.[4,5]

It is well known that electric photometers are ideal for measuring the light transmitted in a parallel direction through a turbid medium.[6] In the present work a red filter was used in such an instrument. Any shift, therefore, in light transmission toward the red end would be reflected in a decrease in density and opacity values of the fluid. Such a decrease is evident after heating the body fluid to 40° or 50°C. This increase in transmitted light may be interpreted as indicating a decrease in the size of the particles in solution. Continued heating above 50° C results in a pronounced increase in optical density indicating a similar increase in the particle size until finally coagulation takes place.

No measurements were made of light scattered in relation to light absorbed by the body fluid. Consequently, it is not certain whether scattering or absorption or a combination of both is responsible for the decrease in transmitted light. Measurements of scattered light are now in progress. However, in other investigations there has been found a consistent parallelism in serological tests between results obtained from measurements of scattered light and those of transmitted light.[7]

The present results are especially interesting if compared with those obtained by Du Noüy using horse serum. His figures indicate no change in light transmitted until the serum is heated to about 56°C at which point there is a pronounced decrease in transmitted light. The consistent preliminary increase in light transmitted after heating to 40° to 50°C, which is found in the body fluid of *Phascolosoma,* does not obtain in horse serum.

Apparently, heating causes the particles in the body fluid in *Phascolosoma* first to decrease in size, perhaps by giving up water, and then to increase in size either by hydration or by aggregation.

The results indicate what might be called 2 critical temperatures for the cell free fluid in *Phascolosoma*: a) 40°C above which heating brings about a decrease in particle size; b) 50°C above which particle size is increased possibly by "intramolecular hydration".[1]

The present method gives a new approach to the study of the phylogenetic relationships of animal body fluids. It is anticipated that a series of investigations covering representative invertebrates and vertebrates will be made with the view to ascertain the evolutionary development of the complex sera of higher animals.

Summary. Cell free body fluid from *Phascolosoma gouldii* was heated in sealed tubes at temperatures from 24° to 90°C. Measurements of light transmitted by the heated fluid indicate that there are 2 critical temperatures for the fluid: a) 40°C above which heating brings about a decrease in particle size; b) 50°C above which particle size is increased.

[4] Burns, D., *An introduction to biophysics,* Macmillan, New York. 580 pp. 1929.

[5] Ostwald, W., and Fischer, M. H., *An introduction to theoretical and applied colloid chemistry.* New York, 266 pp. 1922.

[6] Drabkin, D. L., Photometry and spectrophotometry, in *Medical Physics,* Chicago, 1744 pp., 1944.

[7] Baier, J. G., *Physiol. Zool.,* 1947, **20**, 172.

17017

Observations on the Racial Distribution of Variants of Blood Type rh′.

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Following the demonstration of the existence of 3 major varieties of Rh factors, determining 8 types of human blood, it was found that the incidence of the Rh types differed in different races.[1] It was also found that bloods existed which failed to give a clear-cut positive or negative reaction with one or more of the 3 reagents, anti-Rh_0, anti-rh′, or anti-rh″, indicating the existence of variants of each of the Rh factors.[2,3] In the earlier work these aberrant bloods were designated as belonging to "intermediate" types in order to indicate that the reactions obtained with the reagents were weak or intermediate in intensity. It was observed that the so-called "intermediate" types were particularly frequent among Negroids.[4] The purpose of the present communication is to present the results of a study of so-called "intermediate" types on a larger series of cases, and to discuss the general significance of the findings.

The donors who provided the blood for the present study were individuals who presented themselves at the Blood Bank of the New York University-Bellevue Medical Center of which one of us (L. J. U.) is the Director. They therefore represent a random group who presented themselves consecutively. The tests were carried out in the usual manner, using potent specific reagents of the tube agglutinating variety. For the sake of simplicity, the present paper will be limited only to the results of tests for the rare blood type rh′ and its variants.

In Table I is shown the relative incidence of blood type rh′ among Negroids and Caucasoids. Whereas only 2.7% of the donors who presented themselves belonged to the Negroid race, among those individuals belonging to type rh′ fully 13.9% were Negroids. This indicates that the type rh′ is about 4 to 5 times as frequent among Negroids as among Caucasoids.

As indicated above, some type rh′ bloods gave weak or indefinite reactions when tested with anti-rh′ serum. In Table II is shown the relative incidence of such weakly reacting or "intermediate" bloods among Negroids and among Caucasoids. It will be seen that while only 5% of type rh′ bloods among Caucasoids gave weak reactions with anti-rh′ serum, 27.6% among Negroids gave weak reactions. Therefore, these results on a larger series of cases confirm the previous report concerning the more frequent occurrence of bloods of "intermediate" types among Negroids.[4]

Comment. With regard to the significance of the so-called "intermediate" types, this is best explained by drawing an analogy with the varients of group A. When earlier workers observed that group A bloods fall into two natural subdivisions, namely, those giving strong reactions and those giving weak reactions, they explained these observations on a quantitative basis, believing that weakly reacting bloods contained less agglutinogen A than strongly reacting bloods. From the work of Landsteiner,[5,6] we know the difference in intensity of the reactions of group A bloods does not represent a quantitative difference but instead a qualitative difference. Antibodies and antigens behave in reactions like locks and keys. A key designed for lock X may also open lock Y, but usually not quite

1 Wiener, A. S., Sonn, E. B., and Belkin, R. B., Proc. Soc. Exp. Biol. and Med., 1943, **54**, 238.

2 Wiener, A. S., *Science*, 1944, **100**, 595.

3 Wiener, A. S., Davidsohn, I., and Potter, E. L., *J. Exp. Med.*, 1945, **81**, 67.

4 Wiener, A. S., Unger, L. J., and Sonn, E. B., Proc. Soc. Exp. Biol. and Med., 1945, **58**, 89.

5 Landsteiner, K., and Witt, D. H., *J. Immunol.*, 1926, **11**, 203.

6 Landsteiner, K., and Levine, P., *J. Immunol.*, 1926, **12**, 441.

TABLE I.
Relative Incidence of the Blood Type rh′ Among Negroids and Caucasoids in New York City.

Types of donors	No. of consecutive donors	Caucasoid (%)	Negroid (%)
All Rh types	98,529	97.3	2.7
Type rh′ only	705	86.1	13.9

TABLE II.
Relative Incidence of Weak and Strong Reactions Among Type rh′ Bloods.

Race	No. consecutive type rh′ donors	Strong reactions (%)	Weak reactions (%)
Negroid	98	72.4	27.6
Caucasoid	607	95.0	5.0

as smoothly as in the lock for which it was designed. On the other hand, keys could be designed for lock Y which may or may not open lock X. Similarly, the common anti-A agglutinin clumps A_1 cells somewhat more strongly than A_2 cells, presumably because the configuration of the antibody molecule more closely conforms with A_1 than with A_2. On the other hand, A_2 blood contains other structures which are lacking or different from those present in A_1 blood, as shown by its reactions with anti-O serum. In the same way the existence of type rh′ bloods giving weak or "intermediate" reactions does not necessarily mean that the quantity of rh′ factor in such bloods is smaller than in typical type rh′ bloods, but rather that the rh′ factor is qualitatively different. In support of this interpretation may be cited the observation that among Caucasoids some type Rh_1 bloods which react weakly with anti-rh′ serum are strongly clumped by anti-rh^w (C^w) serum.[7] It may be of interest to mention that despite the relatively high incidence of weak rh′ bloods among Negroids, thus far we have failed to demonstrate the presence of rh^w factor in any blood of Negroid origin in tests on a random series of 105 individuals. No doubt with more intensive and prolonged study, antiserums will be found or produced which strongly clump other "intermediate" rh′ bloods such as described here, while giving weak or no reactions with "typical" rh′ bloods. This same principle would also apply to bloods giving "intermediate" reactions with anti-Rh_o, anti-rh″, anti-hr′, and anti-hr″ sera.

It must be emphasized that one is not justified in giving a specific name to an "intermediate" blood except when a specific antiserum has been found, as in the case of Rh_1^w bloods which contain the special factor rh^w. In the present stage of our knowledge concerning the "intermediate" Rh_o factors, the name D^u is misleading and possibly incorrect, so long as no specific factor or factors corresponding to D^u have been isolated.[8]

Summary. Observations on a large series of blood donors indicate that the rare type rh′ is about 4 to 5 times as frequent among Negroids as among Caucasoids. Moreover, it is found that type rh′ bloods giving weak or "intermediate" reactions are about 5 to 6 times as common among Negroids as among Caucasoids, confirming previous observations on a shorter series of cases. It is pointed out that the existence of bloods giving weak reactions with anti-rh′ serum is more reasonably explained by postulating the existence of qualitative variants of the rh′ factor such as rh'^w, than by attributing the weak reactions to the presence of a smaller quantity of the factor rh′ in the blood. A similar explanation would apply to certain bloods giving "intermediate" reactions with sera anti-Rh_o, anti-rh″, anti-hr′, and anti-hr″.

[7] Wiener, A. S., and Gordon, E. B., in preparation.

[8] Race, R. R., Sanger, R., and Lawler, S. D., *Annals Eug.*, 1948, **14**, 171.

17018 P

Veratramine, an Antagonist to the Cardioaccelerator Action of Epinephrine.

Otto Krayer.

From the Department of Pharmacology, Harvard Medical School, Boston, Mass.

Hitherto studied adrenolytic substances prevent or abolish the vasomotor effect of epinephrine but are unable to modify its effect upon heart rate. Consistent results, obtained in 10 heart-lung preparations of the dog and in 4 spinal cats, show that veratramine,* [1,2] one of the veratrum alkaloids recently reviewed,[3] prevents or abolishes the cardioaccelerator action of epinephrine in doses which do not abolish its vasopressor effect.

In the heart-lung preparation with a total blood volume of 500 to 900 cc[4] the heart rate can be brought to a steady level of between 200 to 260 beats per minute by the continuous infusion of 0.33 to 0.65 cc of epinephrine 1:100,000 per minute. One milligram of veratramine promptly reduces the rate to or near the normal level without modifying the regular sinus rhythm. (Fig. 1).

The veratramine effect can be overcome, at least partially, by sufficiently high concentrations of epinephrine. Atropine in doses up to 10 mg increases heart rate by 10 to 20%, as in the normal heart-lung preparation, and does not abolish the effect. The veratramine action is long lasting. After one milligram it does not begin to wear off within one hour in the heart-lung preparation. In the pithed cat, however, the effect disappears faster.

After pretreatment with veratramine, ex-

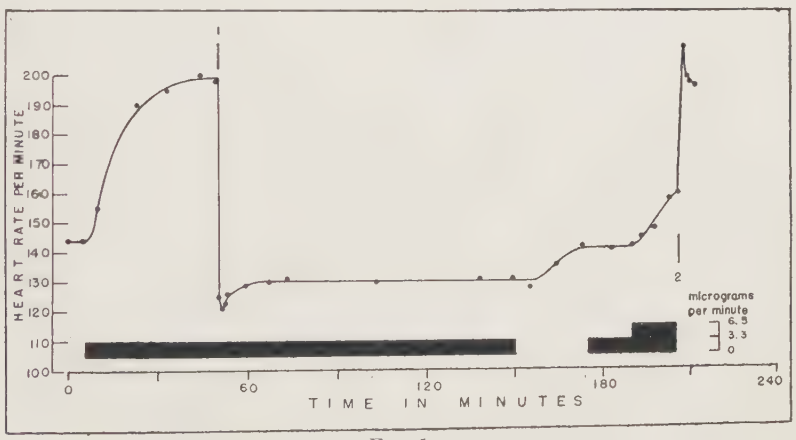

Fig. 1.

Effect of veratramine on cardioacceleration by epinephrine. Heart-lung preparation. Dog, male, 11.6 kg. Total blood volume 800 cc.

Black bar: Continuous infusion of epinephrine tartrate; calibration on right in micrograms of base.

Signal 1: Injection of 1 mg veratramine.

Signal 2: Injection of 500 μg epinephrine (as tartrate) in 5 seconds close to right atrium.

Temperature of blood between 38.6 and 37.6°C.

* Generously supplied by Prof. W. A. Jacobs of the Rockefeller Institute, New York,

1 Saito, K., *Bull. Chem. Soc. Japan*, 1940, **15**, 22.

2 Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, 1945, **160**, 555.

3 Krayer, O., and Acheson, G. A., *Physiol. Rev.*, 1946, **26**, 383.

4 For details of method see Krayer, O., and Mendez, R., *J. Pharmacol. and Exp. Therap.*, 1942, **74**, 350.

TABLE I.

Positive Inotropic Without Positive Chronotropic Action of Epinephrine on Heart in Spontaneous Failure Pretreated with Veratramine. Heart-lung preparation. Dog, male, 9.8 kg. Total blood volume 500 cc. Blood temperature between 39 and 38.5°C.

Time	Heart rate per min.	Systemic output* in cc		Mean pressure			Remarks
		per min.	per stroke	Arterial mm Hg	Pulm. mm water	Right atrial mm water	
1:27 P.M.	163	420	2.6	80	158	27	
1:29	162	420	2.6	80	158	28	Between 1:38 and 3:45 8,5 mg veratramine adm. in divided doses
3:45	105	220	2.1	68	240	76	3:45 heart rate regular, normal sinus rhythm
3:47	105	205	2.0	67	242	77	3:47′ 55-60″ 10 μg epinephrine inj. in 5 sec. (as tartrate)
3:48	105	450	4.3	78	185	19	
3:49	95	420	4.4	78	185	21	
3:51	93	390	4.2	77	198	27	
3:54	96	360	3.8	75	206	34	
3:57	98	330	3.4	72	225	44	
3:58 0- 5″							3:58′ 0-5″ 30 μg epinephrine inj.
10-20″	110			84			
20-30″	127			78	173	10	
30-60″	97			76	185	15	
3:58 total	105	530	5.2†				
3:59	95	440	4.6	76	175	11	
4:01	95	430	4.5	76	180	13	

* Systemic output = total output of left ventricle minus coronary flow.
† Average stroke volume.

perimental heart failure can be relieved by epinephrine without cardioacceleration. (Table I.). In the normal heart-lung preparation 10 and 30 μg (as used in the experiment of Table I) lead to a maximal heart rate increase of at least 20% and 40% respectively; 10 to 15 and 20 to 30 minutes respectively are required for the initial rate to return. It is thus possible to separate the positive chronotropic from the positive inotropic effect of epinephrine. The rate of disappearance of epinephrine appears unchanged, as the positive inotropic action lasts as long as without the presence of veratramine.

Of other veratrum alkaloids studied jervine in a dose of one mg gave an effect similar to 0.1 mg of veratramine, while cevine in a dose of 40 mg was ineffective under the same conditions.

17019

Effect of 1-(3,4, dihydroxyphenyl)-2-Isopropylaminoethanol, (Isopropyl-epinephrine) on the Rhythmic Property of the Human Heart.

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In previous studies[1] a method was described and utilized for the study of the action of drugs on the property of rhythmicity or automaticity of the human heart. The method depends on the fact that it is possible in many individuals especially elderly males, to produce consistently a cardiac standstill of many seconds duration by compression of the carotid sinus. The prolonged cardiac arrest is the result of 2 factors (a) a temporary suppression of the sinus node depriving the heart of its normal pacemaker, and (b) the failure of development of secondary foci of impulse initiation. Following the administration of a variety of unrelated drugs including digitalis, caffeine, coramine, metrazol, barium chloride, calcium gluconate and thyroxin, the cardiac standstill could be consistently reproduced indicating that these compounds were ineffective in stimulating the pacemaking or rhythmic function of the heart. It was found that epinephrine consistently abolished the standstill either by stimulating the normal pacemaker or by initiating new rhythmic foci. This action was found in a number of epinephrine-like compounds (sympathomimetic amines) which were studied. All of the amines which were tested possessed a pressor action. However, it was noted that the increase in cardiac rhythmicity persisted after the blood pressure had returned to normal.

Recently a new sympathomimetic compound has been introduced as a substitute for epinephrine in the treatment of asthma. This compound is the N-isopropyl homologue of epinephrine, 1-(3,4, dihydroxyphenyl) 2-isopropylamino-ethanol. The pharmacological properties of this compound have been described by Lands and his associates.[2] The striking difference in the action of this compound, as compared with epinephrine, is that it does not exert a pressor action, showing usually a moderate depressor effect, especially a lowering of the diastolic pressure.

The study of the effects of a non-pressor compound on the rhythmic property of the heart seemed to be of both practical and theoretical interest. From a practical standpoint, many patients who have heart block or a sensitive carotid sinus, conditions in which a cardiac arrest may occur, have an associated hypertension. The use of a non-pressor compound would be of practical value in such conditions. From a theoretical standpoint, it seemed possible that the mechanism of the increase in cardiac rhythmicity by epinephrine might be clarified. Allen[3] reported that the cardiac effects of epinephrine are largely secondary to the pressor action of the drug. Moe and his associates[4] also concluded that the rise in blood pressure greatly facilitated the induction of rhythmic foci by epinephrine. However, Garb and Chenoweth[5] recently reported observations indicating that a sudden rise in arterial pressure was not necessary

* Research Assistant, University of Southern California, Department of Cardiology, directed by Dr. George C. Griffith.

This study was carried out with the aid of the Dorothy H. and Lewis Rosenstiel Foundation.

1 Nathanson, M. H., Proc. Soc. Exp. Biol. and Med., 1933, **30**, 967; *Arch. Int. Med.*, 1933, **51**, 387; *Arch. Int. Med.*, 1934, **54**, 111.

2 Lands, A. M., Nash, V. L., McCarthy, H. M., Granger, H. R., and Dertinger, B. L., *J. Pharm. and Exp. Therap.*, 1947, **90**, 110.

3 Allen, W. F., *J. Pharm. and Exp. Therap.*, 1934, **50**, 70.

4 Moe, G. K., Malton, S. D., Freyburger, W., and Rennick, B., *Proc. Cent. Soc. Clin. Research*, 1947, **20**, 24.

5 Garb, S., and Chenoweth, M. B., *Fed. Proc.*, 1948, **7**, 220.

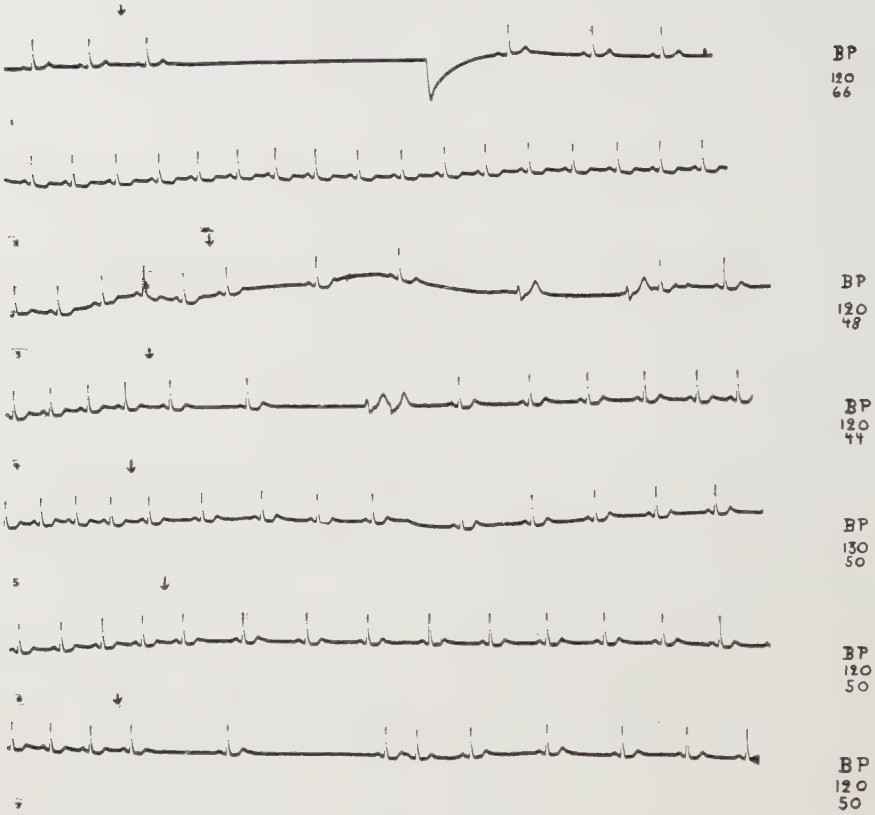

FIG. 1.

Pt. P.L. Strip 1, shows a cardiac standstill of 6 seconds duration induced by pressure on the right cartoid sinus (arrow). Strip 2 shows a sinus tachycardia and depression of the T wave, 5 minutes after the subcutaneous administration of 0.14 mg of isopropylepinephrine. Strips 3, 4, 5, 6, and 7, taken 7, 10, 15, 20 and 25 minutes after the administration of the drug. Carotid sinus pressure (arrow) fails to produce a standstill due to the development of beats arising from the sinus node and occasional beats arising from a ventricular focus (strips 3 and 4). There is a definite lowering of the diastolic pressure.

for the production of ventricular fibrillation by epinephrine during hydrocarbon inhalation.

The effect of isopropylepinephrine on induced cardiac standstill was studied in 14 patients. The procedure was as follows: an electrocardiogram was made showing the cardiac standstill induced by the carotid sinus compression. A blood pressure reading was also made at this time. Isopropylepinephrine was administered subcutaneously in doses of 0.14 to 0.2 mg. Electrocardiograms showing the effect of carotid sinus pressure were made and blood pressure readings recorded, starting two minutes after the administration of the isopropylepinephrine and thereafter at 1 and 2 minute intervals.

Results. The cardiac inhibition induced by the carotid sinus pressure was abolished in every instance following the administration of isopropylepinephrine (Fig. 1 and 2). This effect was noted within 5 minutes after the injection of the drug. A sinus tachychardia was a constant effect. Changes in the contour of the electrocardiogram were frequent, with elevation and occasional flattening and inversion of the T wave. The standstill was abolished by the restoration of the activity of the sinus node or by the initiation of ectopic auricular or ventricular pacemakers. In some instances, multiple rhythmic foci were induced by the drug. The blood pressure responses were as follows: the systolic pressure was unchanged in 6 instances, slightly elevated

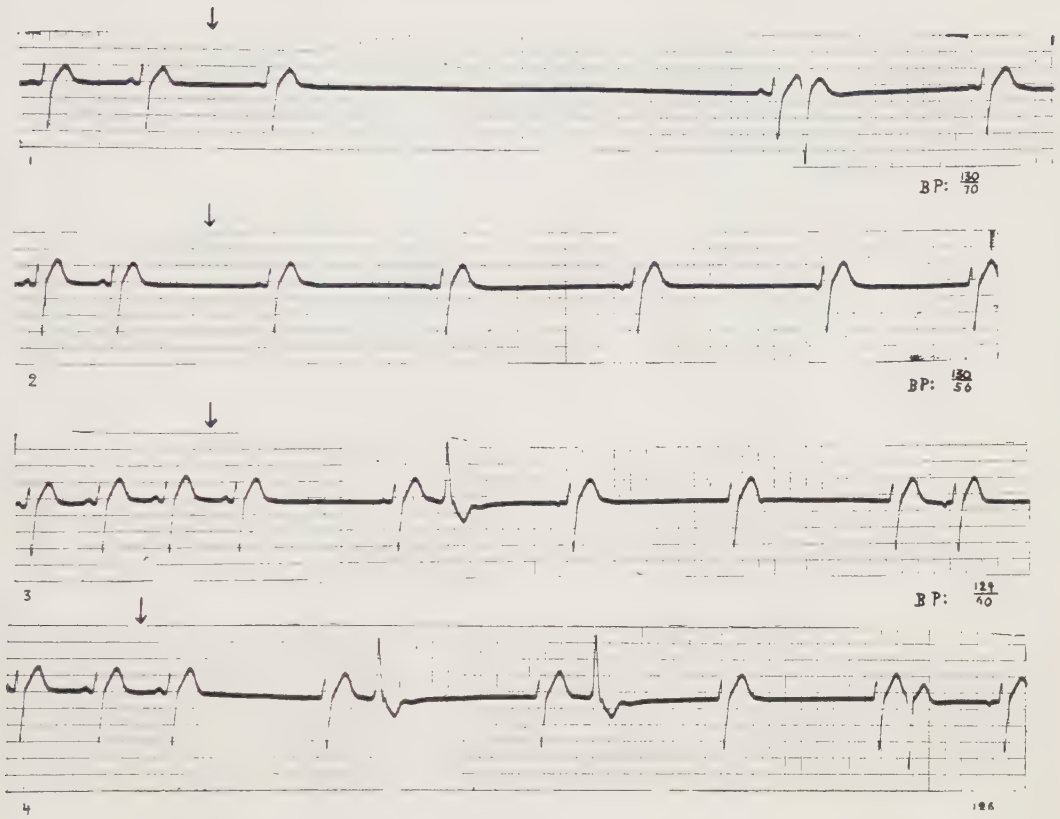

FIG. 2.

Pt. D.C. Strip 1 shows a cardiac standstill of 6.4 seconds duration induced by pressure on the right carotid sinus (arrow). Strips 2, 3 and 4, taken 7, 10 and 15 minutes after a subcutaneous injection of 0.15 mg of isopropylepinephrine. Carotid sinus pressure (arrow) fails to produce a standstill due to the development of beats from nodal and lower auricular foci and of occasional beats from a lower ventricular center.

in four and slightly depressed in 4 experiments. The diastolic pressure was depressed in every instance, in 6 experiments the effect was a reduction of 20 mm or more. There was a widening of the pulse pressure in every case.

Another method for the study of a sympathomimetic action on the heart is the application of the drug to patients with complete heart block. An increase in ventricular rate is an indication of the effectiveness of a sympathomimetic drug on the rhythmic function of the ventricular pacemaker. After a control electrocardiogram was made, isopropylepinephrine 0.2 mg was administered subcutaneously to 5 patients with complete heart block. Electrocardiograms were made at 5 minute intervals for 15 minutes and then at 15 minute intervals. The observations were carried out for 2 hours in 2 instances and one hour in 3 experiments. There was a definite increase in the ventricular rate in every instance. Table I.

Discussion. Isopropylepinephrine, a nonpressor compound, has a potent sympathomimetic action on the heart as indicated by the abolition of a carotid sinus induced cardiac standstill, by the modification of the ventricular complex of the electrocardiogram and by an increase in the ventricular rate in complete heart block. These observations indicate that a pressor action is not essential for the production of an epinephrine-like effect on the heart. The absence of a pressor action may be of practical value in the treatment of heart block or carotid sinus asystole associated with hypertension.

TABLE I.
Effect of Isopropylepinephrine on Ventricular Rate in Complete Heart Block.

Patient	1	2	3	4	5
Initial ventricular rate	54	38	46	31	20
After admin. of Isopropylepinephrine (min.)					
5	57	38	50	37	27
10	61	43	52	48	32
15	60	47	71	54	45
30	57	68	65	50	49
45	60	60	60	46	45
60		50	54	41	44
75			55		
90		38	52		
105			48		
120		38	47		
Max. increase in ventricular rate	7	30	25	23	29

It is probable that isopropylepinephrine has another important advantage in the treatment and prevention of cardiac standstill. In comparing the results of the present study on carotid sinus induced cardiac standstill with previous observations in which epinephrine was used, it appeared that the pacemaker induced by isopropylepinephrine was usually in the sinus node, in lower auricular foci or in the auriculo-ventricular node. There was seldom an excitation of lower ventricular foci. In contrast, epinephrine frequently induced rhythmic foci from lower ventricular centers, and at times multifocal ectopic ventricular beats resembling a pre-fibrillation rhythm occurred. In this connection the recent report of Garb and Chenoweth[5] is of interest. These observers produced ventricular fibrillation consistently in cats during hydrocarbon inhalation by the administration of epinephrine and norepinephrine, while isopropylepinephrine did not induce this arrhythmia under the same conditions. The sudden cardiac failure which occurs in heart block and during surgical operations may be the result of either cardiac standstill or ventricular fibrillation. It is frequently impossible to determine which of these mechanisms is the basis for the sudden cessation of effective cardiac action. The administration of epinephrine, if transient ventricular fibrillation is the mechanism would tend to perpetuate this arrhythmia leading to a fatal termination. It is also known that the treatment of cardiac standstill by the intracardiac administration of an effective dose of epinephrine may lead to ventricular fibrillation. The availability of a potent compound which does not predispose to ventricular fibrillation should reduce greatly the danger of the administration of a sympathomimetic drug in the therapy of sudden cessation of cardiac activity.

Conclusions. Isopropylepinephrine, a nonpressor homologue of epinephrine increases cardiac rhythmicity, as indicated by the abolition of the carotid sinus induced cardiac standstill, and by an increase of the ventricular rate in complete heart block. This drug may be of practical value in the therapy and prevention of sudden cardiac standstill.

The hydrochloride of isopropylepinephrine known as "Isuprel" was used in this study.

17020

Experimental Gastric and Duodenal Ulcer.

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(Introduced by B. P. Babkin.)

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From a review of the literature concerning peptic ulcer one receives the impression that great caution should be exercised in drawing conclusions from the results of experiments in which ulcers have been produced. Although ulcers can be produced readily in certain animals, the conditions necessary to do this are artificial and alter seriously the normal anatomical and physiological relationships of the organs involved. Usually these alterations are far different from any that could possibly occur in human subjects with peptic ulcer. Species differences, too, constitute a hazard in comparing experimental ulcer in animals with spontaneous ulcer in man.

The evidence tends to show that the two outstanding factors in the formation of experimental gastric and duodenal ulcers are the digestive action of acid gastric secretion, and the lowering of mucosal resistance due to local vascular abnormality. The most successful experimental methods for the production of ulcers utilise one or both of these factors.

It is evident that subacute or chronic ulcers resembling those in man are very difficult to produce. While chronic ulcers appear after the establishment of surgical duodenal drainage,[1] it should be realized that this operation has other profound effects, due to the short-circuiting of bile, pancreatic and duodenal secretion past the greater part of the small intestine.

Chronic combined histamine and nitroglycerine stimulation[2,3] is one of the few experimental methods for the production of gastric and duodenal ulcers utilizing the factors both of acid-peptic digestion and of local tissue ischemia in the intact animal. The reported high incidence of lesions in rabbits caused by this technic suggested it to be dependable in an animal that is refractory to other methods.

Code and Varco introduced the method of chronic histamine stimulation. It consists in embedding histamine in a mixture of beeswax and mineral oil before intramuscular injection. This mixture prolongs the action of the contained histamine by delaying its absorption. In this way a large dose of histamine may be injected intramuscularly, with a resultant chronic effect lasting 24 hours or longer. One intramuscular injection daily is, therefore, sufficient to maintain continuous stimulation of gastric secretion.

It was desired, in our laboratory, to use an efficient method for the production of gastric and duodenal ulcers in rabbits. Reports from Wangensteen's laboratory indicated that it was difficult or impossible to provoke ulcers or erosions in rabbits by the chronic action of histamine alone.[3] However, Baronofsky and Wangensteen[4] were successful in producing ulcers in rabbits by means of chronic combined histamine and nitroglycerine stimulation. They reported erosions or ulcers in 9 of 12 rabbits within an experimental period of 6 days. They explained the occurrence of these lesions on the basis of local tissue ischemia (due to the chronic action of nitroglycerine) which rendered the mucosa more susceptible to the digestive action of a highly acid gastric secretion (caused by the chronic action of histamine). In our experiments a longer period was used, with the hope that a higher incidence of ulcers would occur.

1 Mann, F. C., and Williamson, C. S., *Ann. Surg.*, 1923, **77**, 409.

2 Code, C. F., and Varco, R. L., Proc. Soc. Exp. Biol. and Med., 1940, **44**, 475.

3 Hay, L. J., Varco, R. L., Code, C. F., and Wangensteen, O. H., *Surg. Gynec. and Obstet.*, 1942, **75**, 170.

4 Baronofsky, I. D., and Wangensteen, O. H., Proc. Soc. Exp. Biol. and Med., 1946, **62**, 127.

There are conflicting reports[5-7] in the literature concerning the development of gastric and duodenal ulceration following bilateral vagotomy in experimental animals, particularly rabbits. Some investigators have observed the development of ulcerative lesions in more than 30% of vagotomised rabbits, the incidence increasing progressively with the interval of time after vagotomy. Our results in experiments of a similar nature are reported herein. In addition, we investigated the type and incidence of ulcers of the stomach and duodenum of vagotomised rabbits, provoked by the chronic combined sitmulation of histamine and nitroglycerine.

Materials and methods. In order to demonstrate that our beeswax and mineral oil mixture (prepared according to the method of Code) effectively prolonged the action of histamine, experiments were carried out on 2 dogs, one with a Spivack gastrostomy and the other with a metal gastric fistula. The animals were conditioned to the laboratory so that their fasting secretion was minimal. Control specimens were taken, and 0.5 mg histamine injected subcutaneously. The secretion was collected every 15 minutes and volume and pH determined. Subsequently, in each animal, 30 mg of histamine embedded in beeswax and mineral oil was injected intramuscularly. Specimens were collected every 30 minutes for as long as 5¼ hours and the volume and pH of each determined.

A control series of experiments, using 6 rabbits, was performed to determined whether or not daily intramuscular injections of nitroglycerine, embedded in beeswax and mineral oil, produce gastric or duodenal lesions. These rabbits received the stock diet of Purina rabbit pellets, carrots and water. Daily intramuscular injections of 1 mg of nitroglycerine in beeswax and mineral oil were given. Injections were continued for 14 days in survivors and these rabbits were sacrificed on the 15th day for gross and microscopic examination of the involved organs.

It was felt that Code and Varco had demonstrated amply that injections of beeswax and mineral oil alone do not stimulate gastric secretion or produce ill effects in animals. Therefore, no control series of experiments was carried out to confirm this point.

Series I included 32 adult rabbits maintained on the stock laboratory diet. In this series were investigated the type and incidence of lesions of the stomach and duodenum of rabbits produced by the chronic combined action of histamine and nitroglycerine. Daily administration to each rabbit of 30 mg of histamine and 1 mg of nitroglycerine was carried on until death of the animal or until 14 daily injections had been given. Each drug was embedded in beeswax and mineral oil mixture and injected intramuscularly early in the afternoon. Those animals which died during the experimental period were examined as soon as possible, while all survivors were examined on the 15th day.

Series II included 12 adult rabbits in which investigation was made of the incidence of lesions of the stomach and duodenum following bilateral subdiaphragmatic vagotomy. In performing the vagotomy at least 2.0 cm of esophagus was completely cleared of surrounding tissues down to its muscular layer to ensure division of all fibers of both vagus nerves. As a rule these rabbits completely recovered from the effects of the anesthetic within a few hours and were soon lively and eating. Those animals which died were examined as soon as possible after death. The remaining rabbits were sacrificed at different intervals up to 47 days from the time of operation. Survivors were killed by a blow on the neck and autopsies were performed at once.

In Series III 19 rabbits were vagotomised. After a few days they were given daily intramuscular injections of histamine and nitroglycerine, each embedded in beeswax and mineral oil mixture. Injections were carried on for 15 days in survivors, which were sacrificed on the 16th day.

Results and discussion. Fig. 1 shows the results of the experiments performed using Dog No. 1. The results of both parts of the

5 Beazell, J., and Ivy, A. C., *Arch. Path.*, 1936, **22**, 213.

6 Ophuls, W., *J. Exp. Med.*, 1906, **8**, 181.

7 Alvarez, W. C., Hosoi, K., Overgard, A., and Ascanio, H., *Am. J. Physiol.*, 1929, **90**, 631.

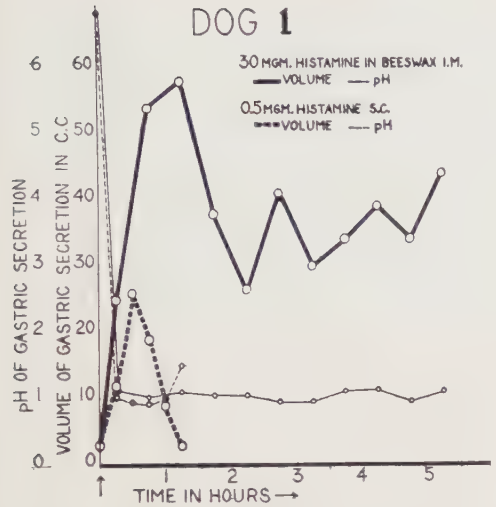

Fig. 1.

Gastric secretory response of dog No. 1. The arrow indicates the time of injection of histamine. Certain volumes are recorded as 15-minute volumes and others as 30-minute volumes.

experiment are plotted on the same graph to emphasize the contrast between the gastric secretory response to a subcutaneous injection of 0.5 mg of histamine in aqueous solution and the prolonged response to an intramuscular injection of 30 mg of histamine embedded in beeswax and mineral oil mixture. Fig. 2 shows the results of similar experiments, using Dog No. 2.

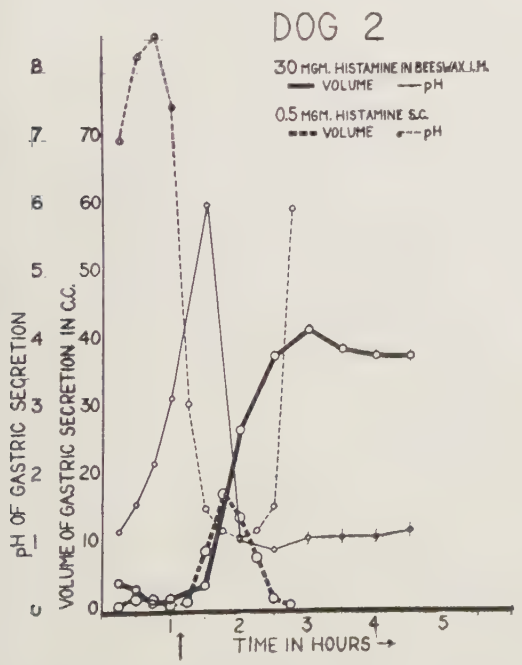

Fig. 2.

Gastric secretory response of dog No. 2. The arrow indicates the time of injection of histamine. Certain volumes are recorded as 15-minute volumes and others as 30-minute volumes.

Following the intramuscular injection of 30 mg of histamine in beeswax and mineral oil our animals showed no evidence of toxic effects of the drug. This was taken as evidence that the large dose of histamine was not absorbed rapidly. In both experiments (Dog No. 1 and Dog No. 2) the rate and acidity of gastric secretion continued at high levels, showing little change in the first few hours. In Dog No. 1 the specimen of gastric secretion obtained 24 hours after the injection still showed a large volume and high acidity of gastric secretion.

These results were taken as proof of the prolonged effects of intramuscular injection of histamine in beeswax and mineral oil. The nitroglycerine control series (6 rabbits) yielded the following results. Two rabbits died of pneumonia during the injection period, one on the 8th and one on the 10th day. Four survivors were sacrificed on the 15th day. Autopsy revealed no gastric or duodenal lesions. This finding was taken as evidence that the chronic action of nitroglycerine alone is incapable of producing ulcerations within a period of 15 days.

Results summarizing the findings in Series I are shown in Table I. In this series, involving 32 rabbits, 12 died during the experimental period. Of these, 9 had perforation of the fundus of the stomach, one had perforation of the first part of the duodenum, one had hemorrhage into the gastrointestinal tract, and one died without a demonstrable lesion to account for its death. Of the 20 rabbits that survived until their sacrifice at the end of the experimental period, 19 had no demonstrable lesion and one had a recent small perforation of the first part of the duodenum with associated localised peritonitis. All the rabbits in this series lost weight during the experiment, so that some were emaciated at the time of death or sacrifice, although food was found in all their stomachs at autopsy.

All perforations of the fundus of the stomach occurred within the first 9 days of the

TABLE I—Series I.

Rabbit	No. daily inj. of histamine and nitroglycerine	Died	Sacrificed	Gross autopsy findings
1	3	X		Perforation, fundus of stomach
2	11		X	No lesion
3	11		X	" "
4	11		X	" "
5	6	X		Perforation of fundus and blood in bowel
6	7	X		Perforation of fundus
7	8	X		Perforation of fundus and blood in bowel
8	14		X	No lesion
9	14		X	" "
10	14		X	" "
11	14		X	" "
12	14		X	" "
13	14		X	" "
14	14		X	" "
15	14		X	" "
16	14		X	" "
17	14		X	" "
18	1	X		Perforation of fundus of stomach
19	2	X		Perforation of fundus
20	3	X		" " "
21	3	X		" " "
22	5	X		" " "
23	11	X		No lesion
24	12	X		Intestinal hemorrhage, no lesion found
25	13	X		Perforation of duodenum
26	14		X	No lesion
27	14		X	" "
28	14		X	" "
29	14		X	" "
30	14		X	" "
31	14		X	Perforation of duodenum
32	14		X	No lesion

experiment; namely, after 1, 2, 3, 3, 3, 5, 7 and 8 injections, respectively. The short time required for the production of these lesions, as well as their microscopic appearances indicate that they were in every sense acute. The perforations of the duodenum, one fatal after 13 daily injections and the other discovered after 14 daily injections, required a somewhat longer time to develop. They were much smaller in size than the gastric perforations.

Generally speaking, the gastric wall near the site of perforation showed very little inflammatory cell infiltration and only moderate edema. All layers of the gastric wall appeared relatively normal to within a short distance of the edge of the perforation, except for scattered superficial mucosal erosions in several instances. The muscularis, at the site of perforation, came to an abrupt end with a small zone of necrosis. The mucosa was absent over a narrow zone about the perforation, leaving the submucosa exposed. This may have been due to retraction of the mucosa after perforation occurred. In some instances minimal leucocytic infiltration was noted, chiefly in the submucosa. Frequently great dilation of blood vessels in the submucosa or subserosal zone was seen.

The preceding brief description of the appearance of sections of gastric wall adjacent to a perforation may be considered representative of the findings in all nine instances of gastric perforation. The gross and microscopic appearances (Table I, Fig. 3, 4) together with the rapid development of these lesions, suggest an acute necrosis of all layers of the gastric wall with digestion of the necrotic tissue resulting in the large, ragged type of perforation. Acute peritonitis, due to the escape of gastric contents into the peritoneal cavity, with or without hemorrhage amply explains the fatal issue in these rabbits.

The two perforations of the first part of the duodenum, one discovered after death of

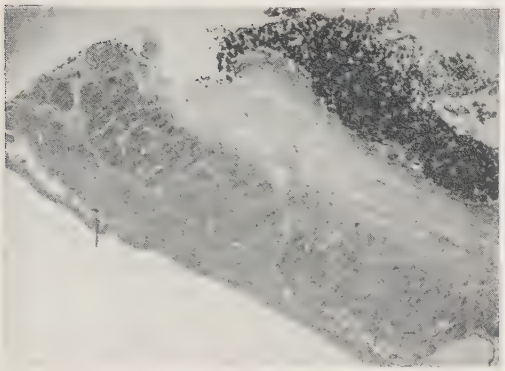

Fig. 3.

Photomicrograph: Rabbit 20, Series I. This shows a section of gastric wall at the edge of the perforation of the fundus. The mucosa shows superficial necrosis, with numerous shallow erosions. The muscularis mucosae and the submucosa are both edematous, so that there is great thickening of this zone of the wall. The submucosa is exposed for a short distance adjacent to the perforation. The muscularis is somewhat edematous and shows a narrow zone of necrosis at the edge of the perforation. The subserosal tissue is edematous and the serosa is necrotic and detached for a short distance. The blood vessels in the submucosa and muscularis are markedly engorged with blood, and the veins, particularly are greatly dilated. There is absence of inflammatory cell infiltration and there is no sign of a repair process.

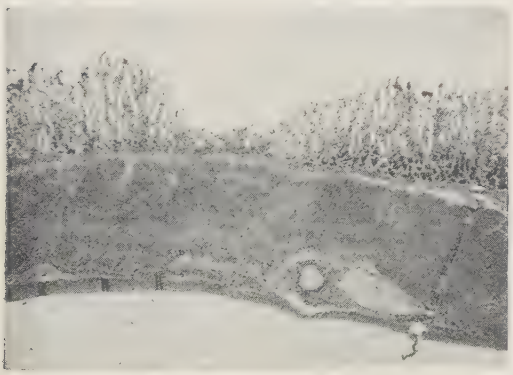

Fig. 4.

Photomicrograph: Rabbit 21, Series I. This shows a section of a gastric mucosal erosion near a perforation of the fundus. There are scattered superficial erosions of necrotic areas of mucosa. The submucosa, muscularis and serosa appear relatively normal apart from marked engorgement and dilatation of the subserosal blood vessels. No apparent inflammatory cell infiltration or evidence of repair is present about the bases of the erosions.

the animal, the other at the time of sacrifice (Fig. 5), were quite similar to one another in microscopic appearance. Sections were cut so as to pass through the involved duodenal wall immediately adjacent to the perforation. There was a moderate degree of acute inflammatory cell infiltration (polymorphonuclear leucocytes and lymphocytes) in the wall of the duodenum surrounding the perforated ulcer. In places there were focal collections of lymphocytes, particularly in the mucosa and submucosa, which were considered to be lymphoid follicles and not part of the inflammatory reaction. The wall of the ulcer contained necrotic tissue with a somewhat homogeneous, eosinophilic appearance. In one instance a thrombosed submucosal artery was seen in the wall of the perforated duodenal ulcer. The ulcer crater was cone-shaped, wider in diameter at the mucosal side of the wall. The duodenal wall at a distance from the perforated ulcer appeared essentially

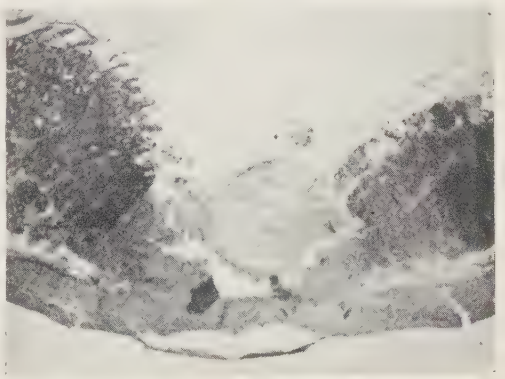

Fig. 5.

Photomicrograph: Rabbit 25, Series I. This is a tangential section through the wall of a perforated duodenal ulcer showing the tissues immediately adjacent to the actual perforation. On each side of the perforation, a short distance away, the wall of the duodenum appears essentially normal. Occasional collections of lymphocytes are present in the mucosa and submucosa representing naturally-occurring follicles and not part of an inflammatory process. The tissue immediately adjacent to the perforation is necrotic, the zone being wider towards the mucosal side and narrower towards the serosal surface. A mass of poorly-stained necrotic debris is lying semi-detached at the level of the mucosa and submucosa. The continuity of the muscularis is seen in this section near the perforation but it is necrotic and no cellular detail is visible. The serosa is necrotic and partially detached. There is a zone of edema of all layers near the necrotic area and a very few polymorphonuclear leucocytes are to be seen in the submucosa at this point. No definite evidence of repair can be detected.

TABLE II—Series II.

Rabbit	Survival time, days after operation	Died	Sacrificed	Autopsy findings
1	47		X	No lesion
2	47		X	" "
3	32	X		Pneumonia
4	20	X		Ear infection
5	43		X	No lesion
6	18	X		Infected wound
7	5	X		Pneumonia
8	38		X	No lesion
9	31	X		Pneumonia
10	31		X	No lesion
11	25	X		Pneumonia
12	4	X		"

normal. No definite evidence of repair was detected in the sections so that these lesions were considered to be acute.

Gross and microscopic examination of the small perforations of the first part of the duodenum suggested the process to have been due to infarction of an area of the duodenal wall followed by the development of an acute perforating ulcer. Although acute in appearance the lesion had developed slowly enough to permit inflammatory cell infiltration about it. This may be contrasted with the large, rapid perforations of the fundus of the stomach which developed in other animals.

In the 21 animals showing no gross lesions of the stomach or duodenum at autopsy, the microscopic appearance of sections of the gastric and duodenal wall was essentially normal.

Table II summarizes the findings in the 12 experiments in Series II. None of the rabbits in this series developed lesions of the stomach or duodenum within periods varying from 4 to 47 days following vagotomy. Seven of the 12 animals died after vagotomy, on the 4th, 5th, 18th, 20th, 25th, 31st and 32nd day, respectively. Of these, 5 died of pneumonia, one of a wound infection and one of an ear infection. The remaining 5 were sacrificed on the 31st, 38th, 43rd, 47th, and 47th days, respectively, after vagotomy.

At autopsy the stomach of each of these animals was found stuffed with food and appeared definitely larger with greater intragastric pressure than was noted during autopsies upon non-vagotomised rabbits. The esophagus was empty in each instance, while the duodenum was either empty or contained a small quantity of bile-stained fluid. No gross lesions were found in other organs apart from those mentioned previously as a cause of death. In each case, the microscopic appearance of the stomach and duodenum was essentially normal. The incidence of pneumonia in this series was notably greater than in animals not subjected to vagotomy. Vagotomy may permit aspiration pneumonia from regurgitation of gastric contents.

It would appear, from the results of this series of experiments, involving 12 vagotomised rabbits, that gastric or duodenal ulcers are not likely to develop following this operation within periods of up to 47 days.

Results summarising the findings in Series III are shown in Table III. Although 24 rabbits were vagotomised in this series, 5 died before the injections of histamine and nitroglycerine were begun. Two rabbits died of pneumonia; one did not recover consciousness following the operation; and the causes of death of the other two are unknown as no autopsies were performed upon them. The three rabbits examined did not have lesions of the stomach or duodenum at the time of death.

Since 5 rabbits died before the injections were begun, it was necessary to exclude them; so that this series actually consisted of nineteen vagotomised rabbits injected daily as described. Of these, nine died before the 16th day of the experiment, at which time the survivors were sacrificed for examination. Of the 9 deaths, 4 were caused by pneumonia, one by infection of an ear, while the other

TABLE III—Series III.

Vagotomised rabbit	No. daily injections	Died	Sacrificed	Autopsy findings
1	15		X	No lesion
2	15		X	" "
3	0	X		Pneumonia
4	15		X	No lesion
5	15		X	" "
6	15		X	" "
7	15		X	" "
8	15		X	" "
9	0	X		Pneumonia
10	15		X	No lesion
11	15		X	" "
12	15		X	" "
13	0	X		(Anesthetic death)
14	0	X		(No autopsy performed)
15	0	X		" " "
16	2	X		Infection—ear
17	5	X		No lesion (emaciation)
18	5	X		Pneumonia
19	6	X		No lesion (emaciation)
20	6	X		Pneumonia
21	9	X		"
22	9	X		No lesion (emaciation)
23	12	X		Pneumonia
24	14	X		No lesion (emaciation)

4 deaths could not be accounted for by any demonstrable lesion apart from marked emaciation. Autopsies revealed no gastric or duodenal ulcerative lesions in any animal in this series of experiments. Microscopic examination of the stomach and duodenum of the animals in this series revealed no abnormalities.

Conclusions. 1. Daily intramuscular injections of 1 mg of nitroglycerine embedded in a mixture of beeswax and mineral oil does not provoke gastric or duodenal ulceration in rabbits within a period of 15 days.

2. Bilateral subdiaphragmatic vagotomy does not produce gastric or duodenal ulceration within periods as long as 47 days in rabbits maintained on a diet of Purina rabbit pellets, carrots and water.

3. Daily intramuscular injections of histamine (30 mg) and nitroglycerine (1 mg) embedded in a mixture of beeswax and mineral oil produce acute, necrotic, perforating lesions of the stomach or duodenum of approximately 30% of rabbits. These lesions are fundamentally different from typical human peptic ulcers.

4. Bilateral subdiaphragmatic vagotomy apparently exerts a measure of protection against the development of acute ulcerative lesions of the stomach and duodenum of rabbits produced by the chronic action of histamine and nitroglycerine.

5. Gastric and duodenal ulcers produced in rabbits by chronic combined histamine and nitroglycerine stimulation are not considered to be suitable lesions for the experimental assessment of ulcer prevention measures.

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Independence of Protamine Titration and Platelet Level in Certain Hemorrhagic Diseases.

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From the Department of Surgery, The University of Chicago.

Following the addition of a standard quantity of heparin to normal blood, the amount of protamine sulfate required to clot this blood within one hour is remarkably constant.[1] Although species differences do exist the amount of protamine required for members of the same species varies to only a slight degree.

This protamine requirement as determined by the protamine titration[2] may be greatly increased in certain pathologic states associated with hemorrhage. Increased protamine requirements in whole blood of patients have been observed in association with acute leukemia, chronic leukemia, some cases of idiopathic thrombocytopenia, secondary thrombocytopenia, aplasia of the bone marrow, and in the toxic states associated with total body X-radiation, nitrogen mustards and aminopterin therapy. All of these conditions are frequently associated with thrombocytopenia. The hemorrhagic complications of these disorders are often temporarily improved or controlled and the protamine titration returned to normal by the administration of toluidine blue, even though the platelet count is unchanged. An increased protamine titration has also been found in bleeding patients with normal platelet counts and normal prothrombin activity.[2] These patients likewise respond to toluidine blue and protamine therapy, and the protamine titrations return to or toward normal.

These and other observations suggest that the increased protamine titration may be due to a defect in the clotting mechanism similar although not identical with that produced by the intravenous injection of commercial heparin. We have been able to demonstrate certain differences between this heparinoid defect and that produced by the intravenous injection of commercial beef heparin in both man and dog.[3] The principal difference appears to be in anticoagulant potency. The bloods of patients and dogs with these disorders rarely inhibited or delayed coagulation of normal bloods. Furthermore, the protamine titration was increased in many of our patients and experimental animals to such an extent that had the increased protamine titration resulted from heparinemia identical with that produced by an injection of commercial beef heparin (Abbott), the blood would invariably have been incoagulable. Incoagulable blood was found in only a few instances. Generally, the clotting time was prolonged only 2 to 4 times the normal values.

Recently Conley, Hartmann and Lalley[4] have observed that normal human plasma could be made more sensitive to the effects of added heparin by removing most of the platelets by centrifugation. They concluded that these plasma data were applicable to the protamine titration and stated that the protamine titration "can be explained by the variation in the platelet concentration alone." The protamine titration, however, is performed on whole blood.

Observations of the type reported by the Hopkins group[4] were investigated by us in

1 Allen, J. G., Moulder, P. V., Elghammer, R. M., Grossman, B. J., McKeen, C. L., Sanderson, M. H., Egner, W. M., and Crosbie, J., *J. Lab. and Clin. Med.*, 1949, **34**, 473.

2 Allen, J. G., Grossman, B. J., Elghammer, R. M., Moulder, P. V., McKeen, C. L., Jacobson, L. O., Pierce, M., Smith, T. R., and Crosbie, J., *S.G.O.*, in press.

3 To be published.

4 Conley, C. L., Hartman, R. C., and Talley, J. S., Proc. Soc. Exp. Biol. and Med., 1948, **69**, 284.

the course of development of the protamine titration. It was found that prothrombin deficient and thrombocytopenic bloods were more sensitive than normal bloods to the effects of added heparin, both *in vitro* and *in vivo*. In this connection the observations of Howell are of interest.[5]

He observed that the effects of amounts of heparin just sufficient to prevent clotting could be overcome by cephalin obtained from brain tissue, recognizing this as a substance also found in platelets. It is possible that other factors may also influence both *in vitro* and *in vivo* heparin sensitivity.

It is doubtful if the increases in the protamine titration can be explained by thrombocytopenia alone. The protamine titration may be normal in severe thrombocytopenia and may be markedly increased in patients with normal platelet counts and prothrombin times.

In order to produce an increase of 0.020 mg in the protamine titration (the smallest increment of increase used) 0.018 mg of liquid beef heparin (Abbott) per ml of human blood was required. This was 2 to 5 times the amount of heparin required to increase the clotting time of whole blood or plasma *in vitro*, and was 25- to 50-fold greater than that required for "platelet-free" plasma.[4] These comparisons were made on normal human blood and plasma and do not necessarily apply to bleeding disorders. Moreover, it has been observed that animals on aminopterin develop an increased protamine titration without thrombocytopenia or an increased *in vitro* heparin sensitivity.[7]

The protamine titration, however, is affected by several factors, most of which influence fibrin formation. Among these factors are: heparin or heparin-like substances, prothrombin deficiency, impairment of the conversion of prothrombin to thrombin, hemophilia, and the absence of fibrinogen. It is possible to set up a very sensitive protamine titration over a narrow range of protamine concentration which can be altered by changes in the platelet count but such a titration would lose its clinical value. The problem of the effect of thrombocytopenia appears to be sufficiently controlled that the results of the protamine titration[1] must be explained on some basis other than platelet deficiency alone.

Twenty-one patients, representing a variety of malignant and benign disorders, had normal platelet counts ranging from 180,000 to 324,000 per cmm but showed mild to marked increase in the protamine titration (0.16 mg to 0.40 mg). On the other hand, eighteen patients with similar bleeding disorders had moderate to marked thrombocytopenia (20,000 to 108,000 platelets per cmm). In these the protamine titration was normal. These observations were repeated 6 to 9 times on some patients. Most patients who had an increased protamine titration also had thrombocytopenia of some degree. In those who responded to toluidine blue the platelet count, before and after partial or complete control of bleeding occurred, was not appreciably altered, Table I.

In Werlhof's syndrome three patients had platelet counts ranging from 2,000 to 60,000 and normal protamine titrations. These did not respond to toluidine blue. Three other patients with platelet counts from 23,000 to 60,000 per cmm and increased protamine titrations (0.16 to 0.20 mg), showed some decrease in bleeding as their titrations returned to normal, but all 3 required splenectomy for complete control of bleeding. In only one of these was the response to toluidine blue impressive. The detailed studies on these and other patients are presented elsewhere.[2]

Comment. These data indicate that the protamine titration may be increased in the presence of normal platelet counts as well as in severe thrombocytopenia. Moreover, the protamine titration can be returned to normal by the administration of adequate toluidine blue without materially altering the platelet count. In addition, thrombocytopenia may

5 Howell, W. H., *The Harvey Lectures*, 1917, **12**, 272.

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TABLE I.
Patients with Increased Protamine Titrations and Low Platelet Counts Before and After Receiving Toluidine Blue.

Patient	Diagnosis	Platelets		Protamine titration*	
		Before toluidine blue, 1000 per cmm	After toluidine blue, 1000 per cmm	Before toluidine blue, mg protamine sulfate	After toluidine blue, mg protamine sulfate
453730	Acute leukemia	190	17	.18	.14
K-a234	" "	20	80	.18	.16
413730	" "	52	24	.18	.16
436363	Acute leukemia Aminopterin Rx	140	24	.18	.16
341183	Hodgkin's Disease Nitrogen Mustard Rx	124	37	.20	.16
395060	" "	83	85	.20	.14
409460	" "	64	37	.20	.14
435361	" "	81	75	.16	.14
453179	" "	19	70	.16	.14
M-a186	Chronic leukemia Spray irradiation	10	16	.18	.16
423054	Cancer Breast P^{32} therapy	100	93	.18	.16
T-a229	Toxic Pancytopenia	7	16	.18	.14
15517	Toxic depression of bone marrow	85	15	.18	.14
H-b32	Menorrhagia	66	72	.16	.14

* Following the addition of a standard concentration of heparin to normal blood a remarkably constant amount of protamine is required to restore clotting. (Laboratory normal is 0.140 mg protamine sulfate (Lilly) per ml blood containing 0.091 mg heparin (Abbott)).

occur without increasing the protamine titration.

The protamine titration is probably increased by factors which directly or indirectly delay or inhibit fibrin formation. Abnormal values in the protamine titration can not be interpreted unless all known clotting factors can be evaluated. The titration can be increased when large quantities of heparin or heparin-like substances are present, but it does not necessarily follow that the factor(s) responsible for the increased protamine titration in these patients was actually heparin or even heparin-like. Some endogenous compounds, not of the heparin family, were found to be mild anticoagulants and were inhibited by both toluidine blue and protamine sulfate.[3] The exact nature of the factor(s) responsible for the defect observed in these patients remains unknown.

Summary. 1. The platelet concentration influences the sensitivity of bloods and plasmas to the effect of added heparin. The anticoagulant potency of heparin is also enhanced by prothrombin deficiency. The potentiated effect of heparin in thrombocytopenic bloods may be due to reduced thromboplastin (cephalin) activity.

2. The protamine titration may be increased when the platelet count is normal and it may be normal in the presence of thrombocytopenia. It is influenced by heparin, heparinoid substances, prothrombin deficiency, hemophilia, and possibly other factors.

3. Increased protamine titrations may occur in bleeding patients who have no other apparent clotting defects, including the whole blood clotting time. Many of these patients cease bleeding when given intravenous toluidine blue. The protamine titration under these conditions can be returned to or toward normal regardless of the platelet level.

17022

Influence of Benzyl-Imidazoline on the Peripheral Circulation of Man.*

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Studies have shown that 2-benzyl-4,5-imidazoline hydrochloride (Priscol hydrochloride-Ciba) has certain properties which may be useful in augmenting the volume of blood flow to the periphery in man.[1] It reverses the hypertensive effect of epinephrine to a hypotensive one through an adrenolytic action.[2] Following the use of this drug in animals, peripheral vasodilatation cardiac stimulation, and an increased cardiac output have been shown.[2] The present study was carried out to determine the effects of the intravenous injection of this drug upon the peripheral circulation in normal individuals and patients with arteriosclerotic obliterative disease and thromboangiitis obliterans.

Methods and materials. Seventy male subjects without evidence of peripheral vascular disease, between the ages of 18 and 45 (average 26) and 10 subjects with organic obliterative disease, between the ages of 35 and 55 (average 51) were studied. Of the latter 7 had arteriosclerosis obliterans and 3 thromboangiitis obliterans. The volume change of the digits was determined using the Cambridge pneumo-plethysmograph[3] and the venous occlusion method.[4] The right or left second toe tip and right or left index finger tip were enclosed in a digital cup from which volume changes were recorded. The collecting cuff was placed at the wrist or ankle. Venous occlusion was ordinarily accomplished by quickly inflating a ribbed-type blood pressure cuff to a pressure of 60 mm of mercury. Lower pressures were often employed in patients with vascular disease. Plethysmograms were standardized so that volume changes were obtained in cu mm per 5 cc of tissue per second. The amplitude of pulsations was recorded in cu mm per 5 cc of tissue. Normal subjects were used as controls for the patients with organic disease. Also control recordings were taken before and at frequent intervals after the injection of the drug so that each subject acted as his own control. Skin temperatures were obtained from a Micromax automatic 4-point recorder which recorded from each thermocouple junction every 2 minutes. Recordings were also made using a Brown Electronik 4-point potentiometer which recorded from each junction every 30 seconds. The readings were accurate to $\pm$ 0.25°C. The accuracy of the instruments was tested using a National Bureau of Standards mercury thermometer which could be read accurately to 0.1°C. The thermocouple junctions were taped to the skin with cellulose tape and inserted into the muscle or subcutaneous tissue of the calf using a specially prepared needle. Determinations were made under standard reproducible conditions. The patients had a normal meal the night before the test. They reported, without breakfast, to the laboratory, dressed in a gown and bathrobe. They rested 45 minutes prior to the test. Their bodies were covered by their robes with no additional blankets during the test (except as described below). The determinations were made in a closed room without draughts. The room temperature did not vary more than $\pm$ 1.5°C during an experiment. Indirect heating of the subjects was produced by covering them with 2 wool blankets (the finger tips and toe tips were exposed)

* The opinions held are those of the authors and not necessarily those of the Veterans Administration.

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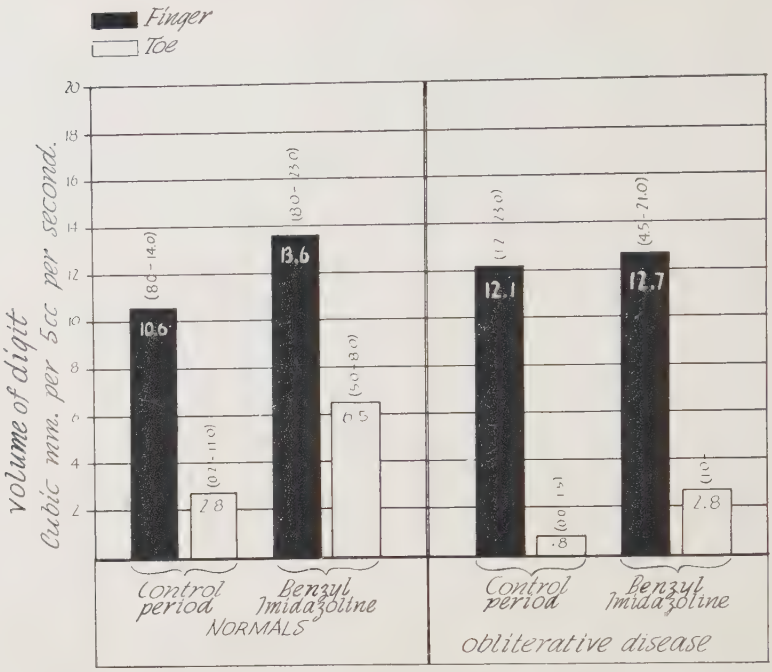

FIG. 1.

Effect of benzyl-imidazoline on the volume of the finger tip and toe tip in 10 normal individuals and 7 patients with arteriosclerotic obliterative disease and 3 patients with thromboangiitis obliterans. The average of 10 determinations was taken on each subject before and 30 minutes after benzyl-imidazoline.

and placing 1 hand and forearm in water at 45°C for 20 minutes. In all experiments 50 mg (5 cc) of benzyl-imidazoline were injected intravenously.

Effect of benzyl-imidazoline on the volume and amplitude of pulsation of the digits. Ten normal subjects and 10 patients with obliterative arterial disease (7 with arteriosclerosis and 3 with thromboangiitis obliterans) were studied (Fig. 1). The room temperature averaged 24°C. In the normal group benzyl-imidazoline increased the volume of the toe tip 1.3 times over that of the control. In the finger tip in this group the change was from 10.6 to 13.6. In patients with obliterative disease the volume of the toe tip increased 2.5 times over that of the control. In the finger tip in this group the change was from 12.1 to 12.7.

Benzyl-imidazoline produced no appreciable change in amplitude of pulsation in the toe tip and finger tip, the values averaging 0.4 before and 0.5 cu mm per 5 cc 30 minutes after in the former and 1.5 and 0.8 cu mm per 5 cc in the latter.

Effect of benzyl-imidazoline on temperatures of deep and superficial tissues. Serial body temperatures were recorded from several body regions before and after injection of benzyl-imidazoline. Ten normal subjects were allowed to rest for 40 to 60 minutes until temperatures varied no more than 0.5°C before the drug was administered. Thirty minutes after injection the temperatures (degrees Centigrade) increased over the resting temperature as follows: forehead 1.0, index finger 1.0, muscle of the calf 0.5, subcutaneous tissue of the calf 0.25, skin of the calf 0.5, skin of the toe 3.75. There was no measurable alteration in the rectal temperature. Thus, benzyl-imidazoline was most effective in increasing the skin flow of blood of the toe.

Effect of indirect heating after benzyl-imidazoline on temperatures of the lower extremity. In 10 normal subjects, at a room temperature which averaged 25°C, benzyl-

imidazoline produced a definite increase in skin temperature which was greatest on the large toe (from 29.5°C to 33.25°C in 56 minutes). The increase in temperature of the skin of the calf, subcutaneous and intramuscular tissue of the calf was less. Indirect heating, after benzyl-imidazoline, produced a further increase in the temperatures, the change being greatest in the large toe. After immersing the arm in water 45°C, the temperature of the large toe rose to 34.4°C. Thus, indirect heating of one arm following benzyl-imidazoline augmented the skin flow to the lower extremity.

Effect of benzyl-imidazoline on skin temperatures following indirect heating. Ten normal subjects were studied. The room temperature averaged 24°C. After 20 minutes of indirect heating the average skin temperature of the dorsum of the left foot was 34.6°C, indicating maximum skin temperature effect ordinarily produced by this procedure. Thirty-two minutes after benzyl-imidazoline the temperature of the foot was 34.7°C. Thus, benzyl-imidazoline after indirect heating had no significant effect on the skin flow to the dorsum of the foot.

The duration of toe temperature changes following benzyl-imidazoline was determined in 10 normal subjects whose resting skin temperatures were less than 32°C. The average increase in temperature was 1.5°C in the first 8 minutes and maximum increase 3°C with a beginning decrease 50 minutes after injection.

Discussion. Benzyl-imidazoline intravenously was effective in increasing the volume of the digits and elevating the skin temperature of the lower extremities in normal individuals and in patients with arterial obliterative disease. The volume changes produced by benzyl-imidazoline were only ¼ as large as the volume changes produced by indirect body heating or arterial occlusion for 5 minutes. It is possible that the intra-arterial injection of this drug would be more effective if tissue fixation is high. The greatest increase in temperature was recorded from the skin of the toe. This effect on skin temperatures occurred within 8 minutes after intravenous injection and after approximately 50 minutes a beginning diminution in effect was noted. A greater increase in temperature and blood flow would probably have been recorded if subjects had been in a state of vasoconstriction prior to the administration of benzyl-imidazoline. The more superficial tissues of the lower extremities showed the greatest elevation in temperature. The temperatures of the rectum, forehead, finger, muscles and subcutaneous tissues of the calf changed relatively little. Benzyl-imidazoline was not effective in raising the skin temperature of the extremities after vasodilatation had been produced by indirect heating. Certain patients with arteriosclerotic obliterative disease responded to the drug with an increased skin flow to the periphery.

In these experiments toxic effects were few. The drug was administered very slowly and was discontinued if signs of toxicity were noted. On two occasions benzyl-imidazoline shock developed with a decrease in rate of blood flow to the periphery, with pallor, sweating and weakness.

The increase in rate of blood flow to the lower extremities is probably brought about by more than one mechanism. Peripheral vasodilatation and cardiac stimulation in animals have been shown.[2] In some of its actions, benzyl-imidazoline resembles epinephrine; in others, histamine or acetylcholine; and still others, ergotamine. Vasodilatation was not prevented by atropine or by sympathomimetic agents and therefore probably was not due to acetylcholine-like or histamine-like action. The sympatholytic action is shown by the effective blocking of the pressor action of epinephrine in experimental animals. Epinephrine following benzyl-imidazoline is an active vasodilator (sympathomimetic antipressor action).[4] Benzyl-imidazoline does not prevent the cardiac stimulation produced by epinephrine. Thus, the increased flow in man may be due to peripheral vasodilatation combined with an increased cardiac output.

Summary. Benzyl-imidazoline given intravenously effectively increased the rate of blood flow to the periphery in normal individuals and in certain patients with arteriosclerotic obliterative disease. The volume change of the digits following venous occlusion and the skin temperatures were aug-

mented to a greater degree in the lower extremities than in the upper. The amplitude of pulsation showed less change than did the skin temperature or volume changes of the digits.

The greatest rise in temperature was seen in the toes. The temperature of the skin of the calf, subcutaneous tissues, and deep muscle of the calf showed a decreasing temperature effect in the order shown. The rectal temperature and temperature of the forehead showed the least change.

Benzyl-imidazoline was not effective in raising the skin temperature of the foot, forehead, or hand after indirect heating of one arm.

Benzyl-imidazoline shock was seen on 2 occasions.

The action of benzyl-imidazoline is complex, and the increased flow to the extremities may result from a combination of effects including peripheral vasodilatation, and, possibly, cardiac stimulation.

17023

Differential Sheep Cell Agglutination Test in Rheumatoid Arthritis.*

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One of the greatest obstacles to the objective study and evaluation of therapy in rheumatoid arthritis has been the lack of a simple and reliable laboratory test for the measurement of rheumatoid activity. Many tests have[1] been proposed in the past and several have proven useful adjuncts to diagnosis and evaluation of a patient but no one test alone is sufficiently significant to serve as sole guide for therapy. Recently Rose, Ragan *et al.*[2,3] have proposed a new simple test for activity in rheumatoid arthritis and have claimed for it a high degree of specificity and reliability. These authors discovered that the serum of patients with rheumatoid arthritis in the active stage contains a substance which agglutinates sheep erythrocytes sensitized with specific amboceptor to much higher titer than normal sheep cells. The test consists in a comparison of the titers to which normal and sensitized sheep cells are agglutinated by patients' serum. The results are expressed as "Differential titer" *i.e.* the algebraic difference between the agglutination titers observed with normal (NS) and sensitized sheep erythrocytes (SS). In patients with active rheumatoid arthritis this differential agglutination titer was found by Rose *et al.* to be "never lower than 16 and usually considerably higher." In other diseases examined (with exception of one case of ankylosing spondylitis) the differential titer was 16 or less.

In order to accumulate data on the validity and possible limitations of this laboratory test, sera from a number of patients were examined in this laboratory. The present paper reports the results from 160 patients living in Northern California, about half of whom had rheumatoid arthritis.

Materials and Methods. The technique described by Rose *et al.*[2] for the differential sheep cell agglutination test was followed in all details. Sera of patients were obtained from freshly drawn venous blood and were tested usually within 18 hours, always within 72 hours of collection. Red blood cells were obtained from a number of different sheep but all gave identical results when tested with the same sera. Special attention was paid to careful preparation of glassware to avoid any

* Thanks are due to many physicians who cooperated in this study by furnishing patients or sera and offering advice.

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2 Rose, H. M., Ragan, C., Pearce, C., and Lipman, M. O., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 1.

3 Editorial, *J. Am. Med. Assn.*, 1948, **138**, 514.

TABLE I.
Differential Sheep Cell Agglutination Test Applied to Sera of 160 Patients.

Clinical diagnosis	No.	"Positive"*		Differential agglutination titer							
		No.	%	0-2	4	8	16	32	64	128	256+
Rheumatoid arthritis											
Marked activity	20	13	65.0	6	1	0	3	6	3	1	0
Mild-moderate activity	37	5	13.5	23	4	6	2	3	0	0	0
No activity	21	0	—	19	2	0	0	0	0	0	0
Other arthritis											
Ankylosing spondylitis	10	1	10.0	9	0	0	0	1	0	0	0
Rheumatic fever	5	0	—	5	0	0	0	0	0	0	0
Miscellaneous	15	0	—	14	1	0	0	0	0	0	0
Other diseases											
Visceral angiitis	5	0	—	5	0	0	0	0	0	0	0
Infectious mononucleosis	7	0	—	7	0	0	0	0	0	0	0
Lymphoma (Hodgkin's, leukemia)	8	0	—	8	0	0	0	0	0	0	0
Hepatitis	24	1	4.1	21	1	1	1	0	0	0	0
Lues	8	0	—	8	0	0	0	0	0	0	0

* "Positive": A differential titer of 16 or over.

false results which might be attributable to remaining traces of soap or detergent.

For sera adsorbed with boiled guinea pig kidney and boiled beef erythrocytes the technique of Davidsohn[4] was followed, except that 1 part of serum was mixed with only 3 parts of 20% suspensions of adsorbent. Because of this, some adsorptions were incomplete.

The diagnosis of rheumatoid arthritis was established in patients on the basis of the opinions of several physicians based on typical clinical observations and laboratory findings. Patients were grouped, relative to the activity of their disease, as "marked," "moderate," and "none."

Results. Sera from 160 patients, 78 of whom carried the diagnosis of rheumatoid arthritis were examined. Table I shows their clinical diagnoses and the results of the differential sheep cell agglutination test carried out on their sera. Among the patients with rheumatoid arthritis, one-third of the group with marked activity had titers in the "normal" range, and two-thirds had differential titers of 16 or more. Almost half of the rheumatoid patients were classified as mildly or moderately active. All of them had pain, progressive limitation of motion, joint changes, increased perspiration, an elevated erythrocyte sedimentation rate and often anemia. Their differential agglutination titers extended over a wide range but only in a few patients were they 16 or higher. In the 21 patients with arrested, inactive rheumatoid arthritis the test was uniformly negative. Eighty-two patients comprised the control group as seen in Table I. Six of the 10 cases of ankylosing spondylitis were definitely active. The single case showing a high differential titer did not appear to be different from the others and had no peripheral joint involvement. No other group showed any tendency toward elevated differential titers except a few patients with hepatitis in whom abnormal globulins might be expected. Two patients with titers of 8 and 16 suffered from severe cirrhosis with reversal of the albumin-globulin ratio.

Thus differential agglutination titers of 16 or more were encountered mainly in patients with active rheumatoid arthritis, but only a small proportion of such patients had high titer sera. The impression was gained that high differential titers occurred predominantly in the most severely ill rheumatoid patients.

It was of interest to determine whether the differential sheep cell agglutination test varied with the treatment and clinical course of the disease. In Table II are shown the results obtained with sera of patients on whom tests were repeated over a period of 3-5 months. In

[4] Davidsohn, I., *J. Am. Med. Assn.*, 1937, **108**, 289.

TABLE II.
Differential Sheep Cell Agglutination Titer in Relation to Clinical Status of Patients Observed During a Period of Time.

Patient	Date	Clinical impression	Therapy	Differential aggl. titer
H	8/24/48	R.A.,* marked activity	Gold	128
	11/ 9	,, ,,	,,	128
	12/21	,, slight improvement	,,	128
	2/ 1/49	,, moderate activity	,,	64
C	9/21/48	R.A., marked activity	,,	16
	12/21	,, ,,	,,	16
	1/25/49	,, moderate activity	,,	8
M	9/21/48	R.A., ,, ,,	,,	16
	12/21	,, ,, ,,	,,	16
	1/25/49	,, ,, ,,	,,	16
L	8/10/48	R.A., ,, ,,	,,	32
	12/21	,, greatly improved	,,	4
	1/25/49	,, no activity	,,	4
Ma	9/21/48	R.A., marked activity	Rest	32
	12/21	,, minimal activity	,,	4
B	9/21/48	R.A., marked activity	Gold	64
	12/21	,, greatly improved	,,	8
	1/25/49	,, questionable activity continued improvement	,,	2

* Rheumatoid arthritis.

these few patients tested and over a short period of time the differential titer did reflect the course of the disease (Table II).

Rose[2] demonstrated by electrophoretic analysis that the substance responsible for agglutination of sensitized sheep cells was a globulin in the beta-gamma fraction of serum. The heterophile antibody present in normal serum is adsorbed by guinea pig kidney. In serum from infectious mononucleosis the heterophile antibody can be removed by adsorption with boiled beef erythrocytes. Table III shows the agglutination of normal and sensitized sheep erythrocytes by adsorbed sera. The normal heterophile antibody of sera from active rheumatoid arthritis was adsorbed by guinea pig kidney. However, neither boiled beef erythrocytes nor guinea pig kidney removed the substance responsible for agglutination of sensitized sheep cells, thus differentiating it from the heterophile antibodies present in sera from normal individuals, from patients with infectious mononucleosis, or from persons who have been injected with horse serum. Thus the differential titer of sera from patients with active rheumatoid arthritis could frequently be increased by adsorbing with boiled guinea pig kidney, prior to the test.

The stability of the substance responsible for agglutination of sensitized sheep erythrocytes was investigated. With sterile sera kept at 4°C both absolute and differential agglutination titers remained essentially stable for 4-20 days. Minimal bacterial contamination quickly destroyed the activity. When stored at —70°C in the dry ice chest in sealed ampoules individual or pooled sera maintained their titer for at least 3 months.

Discussion. The results obtained confirm the claims of Rose *et al.*[2] that there is a substance in the serum of some patients with rheumatoid arthritis which agglutinates sensitized sheep erythrocytes. Adsorption of such sera with boiled guinea pig kidney or beef cells emphasizes the difference between this substance and other components of the serum which agglutinate sheep cells.

There are, however, important differences between the results reported by Rose *et al.* and those observed in the present series. In the New York series all patients with active

TABLE III.
Differential Sheep Cell Agglutination Test Applied to Sera Absorbed with Boiled Beef Erythrocytes and Boiled Guinea Pig Kidney.

Serum	Absorbed with	NS*	SS†	Differential aggl. titer
Normal human	Unabsorbed	8‡	16	2
	Beef erythrocytes	16	16	0
	Guinea pig kidney	0	0	0
Infectious mononucleosis	Unabsorbed	4096	4096	0
	Beef erythrocytes	64	128	2
	Guinea pig kidney	4096	4096	0
Rheumatoid arthritis Marked activity	Unabsorbed	32	2048	64
	Beef erythrocytes	16	2048	128
	Guinea pig kidney	4	2048	512

* Normal sheep erythrocytes.
† Sheep erythrocytes sensitied with specific amboceptor.
‡ Reciprocal of highest dilution giving 2+ agglutination of erythrocytes.

rheumatoid arthritis had differential titers of 16 or more, while in the group of cases in Northern California high differential titers were associated mainly with severe rheumatoid disease and did not occur in many others with obvious activity. Since Rose's method was adhered to in all details these differences in results might represent variations in patients rather than differences in technique. Perhaps the rheumatoid arthritis observed in Northern California is a somewhat milder disease than that on the East Coast. Some cases in the present series, included under the diagnosis of rheumatoid arthritis, despite a typical clinical picture, may actually have had some other disease entity. On the other hand it must be pointed out that on the whole the sensitivity of the test, as carried out in this laboratory, was somewhat less than that indicated in Rose's paper, with both absolute and differential titers at lower levels. It is not clear at this time what technical feature might be responsible for these reported differences.

Rose *et al.* reported that rheumatoid arthritis was the only one of the disorders, sometimes attributed to "hypersensitivity," which produced elevated differential agglutination titers while rheumatic fever and visceral angiitis failed to do so. Our results agree in this respect. One case of ankylosing spondylitis with a high differential titer had, in contrast to Rose's observation, no peripheral joint involvement.

In the group studied the differential sheep cell agglutination test was of value in following the course of patients who initially had high differential titers. Titers observed over a period of 3-5 months in most cases reflected the clinical status. In patients with unequivocal improvement, the differential titer fell long before any change in the erythrocyte sedimentation rate. The test might thus be helpful as an objective aid for the evaluation of the results of treatment.

Summary. Results obtained with the differential sheep cell agglutination test of Rose *et al.* in a series of 160 patients are reported. High differential titers were observed almost solely in sera of patients with severe rheumatoid arthritis. In many others, with milder yet active disease, no significant titers were obtained. Adsorption with boiled guinea pig kidney frequently increased the differential titer of sera from active cases of rheumatoid arthritis. In patients with initial differential titers of 16 or more, tested over 3-5 months, the titers paralleled the clinical course.

17024

Application of Filter Paper Partition Chromatography to Qualitative Analysis of Volatile and Non-Volatile Organic Acids.*

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In attempting to apply filter paper chromatography to organic acids one encounters streaking of many of the acids at the concentrations required for detection by color reaction, spreading or evaporation from the paper of the volatile members of the group, and too rapid migration, essentially with the solvent boundary, of the compounds with low solubility in water. Lugg and Overell[1] have reported a procedure which minimizes streaking and permits separation of many of the non-volatile organic acids. In an attempt to make filter paper chromatography more generally applicable to this class of compounds, a number of salts and derivatives were investigated. Of those tested, the potassium hydroxamate derivative appears most nearly to satisfy the requirements of the procedure. By its use it has been possible to separate most of the common organic acids with chain lengths of about 8 carbon atoms or less.

Methods. The potassium hydroxamates were synthesized from the methyl esters of the organic acids, essentially according to the procedure of Hauser and Renfrow[2]. Separate solutions of hydroxylamine hydrochloride (2.4 g or 0.033 mole in 15 ml methyl alcohol) and potassium hydroxide (2.8 g or 0.05 mole in 10 ml methyl alcohol) were prepared at the boiling point of methyl alcohol. Both were allowed to cool to 30-40°C, the one containing alkali was added with shaking to the hydroxylamine solution, and the mixture was placed in an ice bath for 5 minutes to permit complete precipitation of potassium chloride. To this mixture was added with thorough shaking, 0.017 mole of the methyl ester of the organic acid (or one-half that quantity of the methyl ester of a dicarboxylic acid). The mixture was filtered immediately with suction, and the precipitate rinsed with a few ml of methyl alcohol. The precipitate remaining in the funnel was discarded. Methyl alcohol was added to the filtrate to make a final volume of 30 ml if 0.017 mole of the ester was added. When 0.0085 mole was used, the hydroxamate was concentrated on the steam bath to a final volume of 15 ml, and for some of the hydroxamate derivatives, especially of the dicarboxylic acids, it was necessary to evaporate off most of the methyl alcohol and add water to obtain solution of the precipitate which formed. No determinations were made of the yields of the hydroxamates in their synthesis from the acid.

Details of the apparatus used and the technique for partition chromatography have been described.[3] For the chromatograms, 5 microliters (3×10^{-6} mole) of the potassium hydroxamate solution were used. A saturated solution of ferric chloride in n-butyl alcohol saturated with water was used as the color developing reagent.

Results. A number of solvents were found to be satisfactory for separating short-chain monocarboxylic acids, and R_F values for a number of such acids are given in Table I. Acids containing more than one carboxyl group frequently streaked, or gave numerous spots, or did not move sufficiently far from the initial position to permit a good separation in many of the solvents which were tried.

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2 Hauser, C. R., and Renfrow, W. B., Jr., *Organic Syntheses*, 1939, **19**, 15.

3 Dent, C. E., *Biochem. J.*, 1948, **43**[2], 169.

TABLE I.
R_F Values of Hydroxamate Derivatives of Organic Acids in Various Solvents on Whatman No. 1 Filter Paper.

Hydroxamate	Solvent						
	n-Hexyl alcohol	n-Amyl alcohol	n-Butyl alcohol	sec-Butyl alcohol	Methyl ethyl ketone	Isobutyric acid	Phenol
Formic	.06	.12	.40	.54	.22	.45	.57
Acetic	.23	.35	.51	.63	.40	.57	.70
Propionic	.43	.56	.68	.78	.61	.68	.78
Butyric	.63	.71	.79	.87	.75	.74	.80
Valeric	.73	.78	.86	.90	.84	.83	.84
Caprylic	.86	.88	.90	.91	.91	.87	.90
Pelargonic	.84	.85	.91	.90	.91	.88	.90
Capric	.89	.89	.92	.90	.90	.92	.95
Benzoic	.69	.73	.82	.86	.83	.79	.85
Phenylacetic	.73	.76	.83	.83	.84	.75	.86
Lactic	.14	.23	.42	.53	.28	.50	.66

TABLE II.
R_F Values of Hydroxamate Derivatives of Organic Acids in Phenol and in Isobutyric Acid on Whatman No. 1 Filter Paper.

Hydroxamate	Solvent	
	Phenol	Isobutyric acid
Oxalic	.14, .40	.23, .28, .32
Malonic	.11, .23	.19, .32
Succinic	.40, .72 orange	.45, .52 orange
Glutaric	.47	.37, .52
Adipic	.54, .67	.44, .60
Pimelic	.60, .73	.52, .69
Azelaic	.63, .74	.66 streaked
Sebacic	.89	.74, .89
Citric	.09, .23	.20, .29
Tartaric	.10	.19
Pyruvic	.59, .86	.54, .62 orange, .73

The most satisfactory solvents of those tested for these acids were isobutyric acid and phenol. R_F values for a number of these acids and for pyruvic acid are given in Table II, and a diagram of a two dimensional chromatogram is given in Fig. 1.

Discussion. Converting the organic acids to their hydroxamate derivatives increases the polarity, decreases the volatility, and makes possible the use of a convenient and reasonably sensitive color reaction for development of the chromatogram without increasing the molecular weight to such a degree that solubility differences between members of a homologous series are seriously diminished. In general, the color reaction is sufficiently sensitive to detect on the finished chromatogram a spot containing in the order of 10^{-7} mole of acid, assuming a quantitative conversion of the ester to the hydroxamate in the preparative procedure, while use of a mixture containing more than about a milligram of any one component is likely to lead to spreading or streaking due to the overloading of the paper. With proper controls, a rough quantitative estimation may be made of the amount of acid in a spot by judging from the size of the spot and the intensity of the color.

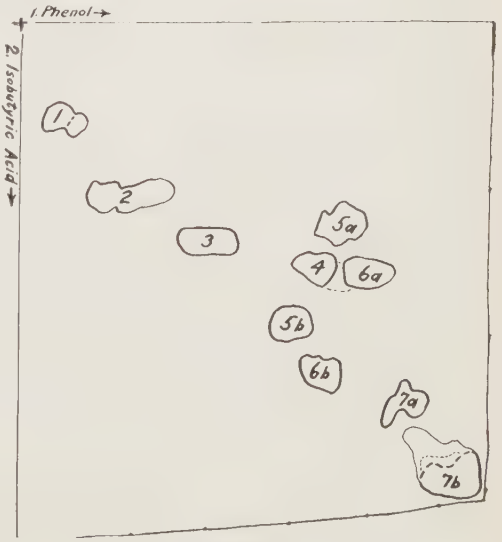

FIG. 1.
Diagram of a two dimensional chromatogram of hydroxamate derivatives of organic acids. The hydroxamates were applied at the point indicated by the cross. Phenol was used as the first solvent and isobutyric acid as the second. The numbers refer to the acids as follows:

1. tartaric
2. malonic
3. succinic
4. lactic
5. adipic
6. pimelic
7. sebacic

Disadvantages in the use of the hydroxamate derivatives include the manipulations involved in their preparation and the multiple spots obtained from the acids with multiple functional groups. The multiple spots are presumably due to failure to carry the preparative reactions to completion.

The colors in the developed chromatogram are relatively stable, although continued exposure to strong light causes a loss of contrast, principally by action on the ferric chloride background. Intense spots of the ferric hydroxamates tend to spread to other papers in contact with them, so that in filing it is well to insert blank sheets of paper between the chromatograms.

One dimensional chromatogram may be made on filter paper previously impregnated with ferric chloride, in which case the chromatogram may be examined visually at any stage of the development, but better results are usually obtained by use of non-impregnated paper.

Summary. A procedure for the application of filter paper partition chromatography to the separation of both volatile and non-volatile organic acids with chain lengths of about eight carbons or less is described. The potassium hydroxamate derivatives of the organic acids are prepared by reacting the methyl ester with about a two-fold excess of a mixture of potassium hydroxide and hydroxylamine in methyl alcohol. The hydroxamate derivatives obtained from about 10^{-6} mole of each of the esters are applied to the filter paper, the chromatogram developed with suitable solvents, and then sprayed with ferric chloride to make the derivatives visible as purple spots on a yellow background. Isobutyric acid and phenol gave the best results of the solvents tried for two-dimensional chromatograms of the dicarboxylic acids. R_F values for a number of hydroxamate derivatives are given for several solvents.

17025

Destructive Action of Human Cancer Extracts on Red Blood Cells *in vitro*.*

LUDWIK GROSS.

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It was recently observed in this laboratory that centrifugated or filtered extracts prepared from spontaneous mouse mammary carcinomas hemolyze mouse erythrocytes *in vitro*.[1]

Experiments reported in this study were designed with the purpose of determining whether extracts prepared from human cancer also exert a destructive action on red blood cells *in vitro*.

Materials and methods. Tumor extracts. Sterile specimens of tumors were obtained from the operating room.† The tumor tissue was weighed, cut with scissors into small pieces, then ground thoroughly for several minutes in a porcelain mortar, 0.85% solution of sodium chloride being added to obtain cell suspensions varying in concentration from 20 to 30%. The suspensions thus obtained were cleared from cells by 2 successive centrifugations at 5,000 t.p.m. for 10 minutes each; the final supernatant fluid was then used, and designated "tumor extract." In several cases, tumors of hard, fibrous consistency had to be ground into fine cell suspensions in a Latapie Grinding Apparatus (Thomas), and then centrifugated.

Cell suspensions prepared from human

* Published with the permission of the Chief Medical Director, Department of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or conclusions drawn by the author.

[1] Gross, L., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 292.

† Most of the tumor specimens were obtained from the operating room of our hospital (Veterans Administration Hospital, Bronx, N. Y.); 10 of the 12 specimens of human breast carcinomas were obtained from the Memorial Hospital, New York City.

TABLE I.
Action of Cell-Free Human Tumor Extracts on Human RBC *in vitro*.

Tumor specimen			Blood group of patient	RBC used for test	Results of the RBC test *in vitro*	
No. of test	Specimen diagnosis				Hemolysis after 48 hr incub. 37°C	Agglutination after 24 hr incub. 37°C
1.	Carcinoma, breast	(F)	O pos.	O pos.	+	0
2.	" "	(F)	O pos.	O pos.	+	0
3.	" "	(F)	O pos.	O pos.	+	sl†
4.	" "	(F)	B pos.	B pos.	+	+
5.	" "	(M)	A pos.	A pos.	+	0
6.	" "	(F)	O pos.	O pos.	sl†	sl†
7.	" "	(F)	O pos.	O pos.	0‡	+
8.	" "	(F)	O neg.	O neg.	sl†	0
9.	" "	(F)	ND*	O pos.	+	sl†
10.	" "	(F)	ND*	O pos.	+	0
11.	" "	(F)	ND*	O pos.	+	+
12.	" "	(F)	ND*	A pos.	+	+
				B pos.	+	+
13.	Carcinoma, stomach	(M)	A pos.	A pos.	0	+
14.	" "	(M)	O neg.	O neg.	+	sl†
15.	" "	(M)	A pos.	A pos.	+	+
16.	" "	(M)	A pos.	A pos.	+	sl†
17.	" "	(M)	B pos.	B pos.	+	0
18.	" rectum	(M)	B pos.	B pos.	sl†	0
19.	" antrum	(M)	O neg.	O neg.	0‡	sl†
20.	" "	(M)	A neg.	A neg.	+	0
21.	" metastatic (primary undetermined)	(M)	A pos.	A pos.	sl†	sl†

* ND—Blood group not determined.
† sl Slight.
‡ Minimal hemolysis noticed.
(F) female.
(M) male.

breast cancer formed a fatty layer on the surface of the supernatant fluid following centrifugation; this fat had to be separated from the extract by passing the supernatant fluid through a sterile voile filter.

In a few instances, apparently infected tumors such as carcinoma recti, or that of the antrum, were used for the tests. To assure bacterial sterility, extracts prepared from such tumors were passed through a Seitz filter, and the filtrate was used for the tests. In addition, filtrates, as well as centrifugated extracts, were prepared also from several other bacteriologically sterile tumors.

The entire procedure followed in the preparation of the extracts was aseptic, and the extrates, or filtrates, used for the tests were found to be bacteriologically sterile, as evidenced by negative inoculations of ordinary culture media.

Normal Tissue Extracts. Control experiments were performed with cell-free extracts prepared from various freshly obtained and sterile human tissues: specimens of healthy muscle were obtained from patients in whom a limb was amputated; specimens of thyroid gland were obtained from patients in whom thyroidectomy was performed because of thyreotoxicosis; specimens of hypertrophic, but otherwise normal, breast gland were obtained from 5 males operated on for gynecomastia, and from one female in whom a plastic breast operation was performed; a specimen of placenta was obtained from a young woman in whom Caesarean Section was performed; finally, 2 small specimens of apparently normal liver were obtained from patients in whom a diagnostic liver biopsy was performed. From these various tissue specimens, centrifugated, cell-free, and bacteriologically sterile extracts were prepared in a manner identical with that described for the preparation of the tumor extracts.

Red Blood Cells (RBC). Freshly drawn, oxalated red blood cells from healthy human donors, as well as in some instances also from

guinea pigs, rabbits, chickens, and mice, were washed twice at 2,500 t.p.m. for 3 minutes each, and final one per cent suspensions of the red blood cells in 0.85% solution of sodium chloride were prepared. The washed red blood cell suspensions were freshly prepared for each test. In all instances, except when indicated otherwise in Table I, human erythrocytes of the same type were used as that of the patient from whom the specimen had been removed.

Technic of the Test. The test was performed in the following manner: one cc of the 1% RBC suspensions was mixed gently in a small (10 x 75 mm) tube with doses of the tumor, or normal tissue, extracts varying from 1 to 0.25 cc, and placed in an incubator at 37°C for 48 hours. The presence or absence of agglutination was determined after 24 hours of incubation at 37°C; agglutination, if any present, was graded "slight," "1+," and "2+." The presence or absence of hemolysis was determined by inspecting the tube, without previous centrifugation, after incubation at 37°C for 48 hours; hemolysis present only over the sediment was graded "slight"; if hemolysis was stronger so as to diffuse in the supernatant fluid to a level extending at least for 1 cm above the sediment, it was graded "1+."

Experimental. "Agglutinating" Action of the Human Tumor Extracts on RBC in Vitro. The curious clumping action of tumor extracts on red blood cells, observed in experiments with transplanted mouse carcinomas,[2] was reproduced in some of the tests performed with human cancer extracts mixed with human erythrocytes. The clumping potency of the tumor extracts was not a constant phenomenon (Table I). In some instances, it occurred promptly; in others, only very slightly, and with a considerable delay, or it was missing entirely. Even in the more pronounced cases, however, the examination of the sediment under the microscope failed to show a typical picture of agglutination (Fig. 1 to 3). The clumping of the cells was usually only slight, if at all present, and was far from being uniform; only some of the cells were affected;

FIG. 1.
Agglutinating action of human breast carcinoma extract on human erythrocytes *in vitro* after 24 hours of incubation at 37°C. Mag. ×67.

accumulation of either debris, or some unidentified precipitated masses could also be noticed. The interpretation of these findings was not facilitated by the observation that frequently a slight precipitation occurred also in control tubes containing tumor filtrates only, without erythrocytes, and incubated at 37°C for 24 hours.

In 8 experiments the tumor extracts were passed through a Seitz filter and the filtrates were mixed with the erythrocytes; in all these cases the agglutinating potency of the extracts was not diminished by filtration. In fact, the agglutinating potency of the filtrates appeared to be somewhat more distinct than that of the centrifugated extracts.

In 10 experiments the tumor extracts were heated to 56°C for 30 minutes in a water bath prior to mixing them with red blood cells. All such preheated extracts were found to have lost their ability to agglutinate erythrocytes *in vitro*.

A series of experiments was performed in which the human tumor extracts were mixed with erythrocytes of mice, rats, guinea pigs or chickens. In all these instances in which such extracts were found to agglutinate human red blood cells, they were also found to exert a similar agglutinating action on the erythrocytes of the various different species of the animals tested.

Hemolytic Action of Human Tumor Ex-

[2] Gross, L., *J. Immunol.*, 1948, **59**, 173.

FIG. 2.
A slight clumping action of an extract from a human stomach carcinoma on human erythrocytes after 18 hours of incubation at 37°C. Mag. ×67.

FIG. 3.
Human erythrocytes, 1% suspension, after 24 hours incubation at 37°C. Control slide. Mag. ×67.

tracts on Human RBC in Vitro. Most of the extracts prepared from human tumors were found to hemolyze (Fig. 4) human erythrocytes *in vitro* after an incubation for 48 hours at 37°C (Table I). Control tubes containing human red blood cell suspensions in physiological saline solution, and incubated simultaneously under otherwise identical conditions, remained unaltered.

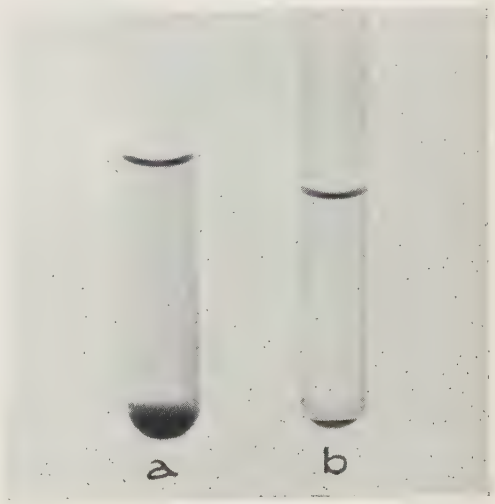


FIG. 4.
a. "Slight" hemolysis resulting from exposure (48 hours at 37°C) of human erythrocytes to a filtered extract prepared from human breast carcinoma. b. Control tube containing erythrocytes suspended in physiol. saline solution; no hemolysis.

In 8 experiments the tumor extracts were passed through Seitz filters, and the filtrates, as well as the corresponding centrifugated extracts, were tested for their hemolytic potency. In 3 instances the tumor extracts lost their

hemolytic potency after filtration; in 4 instances, the hemolytic potency of the extract was substantially diminished, but detectable, after filtration. Finally, in one instance, the hemolytic potency of the extract was not diminished after filtration.

In 10 successive experiments, human tumor extracts were heated to 56°C for 30 minutes in a water bath, prior to mixing them with the red blood cells. In all instances the hemolytic potency of these extracts was destroyed by heating.

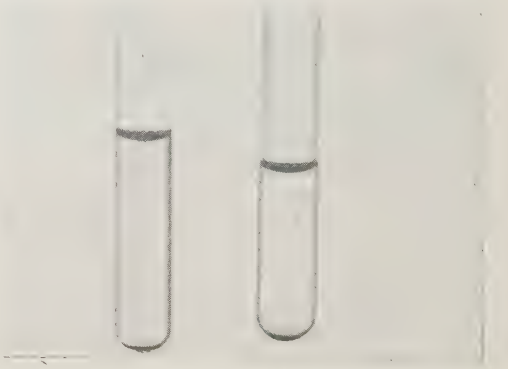
In 4 experiments, centrifugated tumor extracts, separated from the tumor cells, were incubated at 37°C for 4 hours, and then mixed with the erythrocytes. The potency of these pre-incubated extracts did not appear to be diminished, as compared with the corresponding control samples of fresh tumor extracts. This was in marked contrast to similar experiments showing that mouse tumor extracts lose their hemolytic potency when separated from the tumor cells, and incubated at 37°C for a few hours.[2]

An attempt was made to determine whether human tumor extracts would also hemolyze red blood cells of other species of animals. Experiments dealing with this question encountered, however, a rather unexpected difficulty; thus, it was found that washed mouse erythrocytes undergo spontaneous hemolysis when suspended in physiological saline solution, and incubated at 37°C for 12 to 24 hours; erythrocytes of rabbits, and to a lesser degree those of guinea pigs, were also found to be not suitable for a long incubation at 37°C.

In a few cases only, using potent human tumor extracts, was it possible to observe a slight hemolytic action of the human tumor extracts on the red blood cells of mice, rabbits, and guinea pigs, occurring comparatively promptly, and at a time when the control tubes with the respective erythrocytes suspended in physiological saline solution did not yet show any trace of hemolysis. The washed chicken erythrocytes appeared to be more resistant to spontaneous hemolysis when incubated at 37°C, and for that reason were found to be better suitable for these tests than the red blood cells of guinea pigs, rabbits or mice. In a few instances it was found that human tumor extracts slightly hemolyzed chicken erythrocytes after incubation for 48 hours at 37°C.

Experiments with Extracts Prepared from Non-Malignant Tumors. Four experiments were made with extracts prepared from non-malignant human tumors, *i.e.* from 3 prostatic adenomas (one centrifugated and 2 filtered extracts), and from a uterine myoma. In all instances washed erythrocytes of the same type were used for the tests as the type of the patient from whom the specimen had been removed. The results were as follows: the 2 filtrates prepared from the prostatic adenomas agglutinated the red blood cells after 24 hours of incubation at 37°C; the other 2 extracts did not agglutinate the erythrocytes. All 4 tests were negative for hemolysis after 48 hours of incubation at 37°C; after additional 24 hours of incubation, however, the prostatic adenoma extract, one of the 2 prostatic filtrates and the uterine myoma extract, produced a slight hemolysis of the corresponding erythrocytes.

Action of Normal Human Tissue Extracts on Human RBC in Vitro. Sterile, cell-free, centrifugated extracts freshly prepared from various normal human tissues, were mixed with 1% suspensions of washed human erythrocytes of the same type as that of the patient from whom the respective specimen



a b

FIG. 5.

Exposure of human erythrocytes (48 hours at 37°C) to the action of an extract prepared from human breast gland (gynecomastia). a) Extract from breast gland (1 cc), and RBC. No hemolysis. b) Physiol. saline solution, and RBC. Control tube. No hemolysis.

TABLE II.
Action of Cell-Free Extracts from Normal Human Tissues on Human RBC *in vitro*.

Organs tested	Blood group of patient	No. of individual extr. tested	Blood group or RBC used	No. of extr. causing hemolysis	No. of extr. causing agglut.
Muscle	A pos.	4	A pos.	0	1*
"	A neg.	1	A neg.	0	0
"	O pos.	2	O pos.	0	0
"	O neg.	1	O neg.	0	0
Placenta	B pos.	1	B pos.	0†	0
Thyroid	B pos.	1	B pos.	0	0
"	O pos.	1	O pos.	0†	0
"	O pos.	1	O pos.	0	0
"	AB pos.	1	AB pos.	0	0
Breast (F)	A pos.	1	A pos.	0	0
" (M)	O pos.	4	O pos.	1*	0
Liver	A neg.	1	A neg.	0†	0
	B pos.	1	B pos.	0	0

Each extract was prepared from a different patient. The agglutination was read after 24 hours of incubation at 37°C. The hemolysis was read after 48 hours of incubation at 37°C.
* "Doubtful."
† Slight hemolysis after 72 hours of incubation at 37°C.

had been removed: these mixtures were then incubated, at 37°C, for 48 hours (Fig. 5). There was only one instance of a "questionable" agglutination (Table II.); all other sediments were negative for clumping when inspected after either 24 or 48 hours of incubation. Of the 21 individual extracts tested, only one produced a minimal hemolysis after 48 hours of incubation; in 3 additional instances, a slight hemolysis resulted after 72 hours of incubation at 37°C.

Discussion. In previous experiments,[1,2] mouse mammary carcinoma extracts were found to hemolyze mouse erythrocytes, *in vitro*, after 2 to 3 hours of incubation at 37°C. Agglutination of the red blood cells by mouse tumor extracts was found to be less frequent, unless transplanted tumors were used for the preparation of the extracts.[2] Both observations have been recently confirmed by Zimmerman[3] and Salaman.[4]

At first, similar experiments performed with human tumor extracts in this laboratory gave negative results, except for occasional agglutination[5] (Fig. 6); in these preliminary tests the readings were made after a brief incubation at 37°C, not exceeding 3 or 6 hours. In the case of experiments with mouse tumor extracts a longer incubation was not necessary, and for that reason a similar procedure was followed in these tests dealing with human cancer. Later on, however, the human tumor extracts were allowed to act on human erythrocytes for longer periods of time; this modification in the technic of the test lead to positive results: agglutination, and hemolysis were found to result frequently. Even after a prolonged incubation, however, the hemolytic action of the human tumor extracts on human erythrocytes was found to be somewhat less pronounced than the similar action of mouse carcinoma extracts on mouse red blood cells.

The hemolytic, and particularly the agglutinating actions of the extracts prepared from various human tumors were far from constant; in some instances the hemolysis and/or agglutination occurred promptly and to a pronounced degree. In other cases, the destructive action of the tumor extracts was slight, and delayed. Since, however, the various extracts were prepared from different tumors, it was reasonable to expect differences in their ability to destroy the erythrocytes. Similar differences in the potency of the individual tumors were also observed in experiments dealing with mouse carcinomas.[2]

The question immediately arose whether cell-free extracts prepared from normal human tissues would also exert a similar destructive

[3] Zimmerman, H. M., (Montefiore Hospital, Bronx, N. Y.), personal communication to the author.

[4] Salaman, M. H., *Brit. J. Cancer*, 1948, **2**, 253.

[5] Gross, L., unpublished experiments.

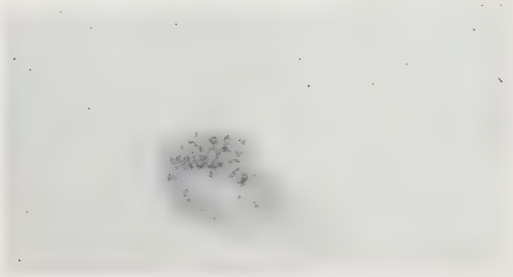


FIG. 6.
Agglutination of human erythrocytes by a human carcinoma (stomach) extract after an incubation, in test tube, at 37°C, for 6 hours. A drop of the sediment was then placed on a slide. The agglutination is visible macroscopically. Mag. ×1½.

action on human erythrocytes; this point was of particular interest in view of the fact that preincubated extracts from certain mouse tissues,[6] as well as *slices*[7,8] of normal animal or human tissues, had been found to hemolyze red blood cells *in vitro* after a prolonged incubation at 37°C. Accordingly, a series of experiments was performed with freshly prepared cell-free extracts from various human organs. With insignificant exceptions, the results of these tests were negative.

Anemia is a frequent associate of cancer. Some of the tumors, such as carcinomas of the intestinal tube, may ulcerate and bleed, giving apparently sufficient reason for the resulting anemia. In other instances, however, no bleeding occurs, and yet anemia also eventually develops. The exact cause of the progressive anemia so frequently encountered also in non-bleeding, and not ulcerated cases of cancer, has been a matter of controversy.

Experiments reported in this paper suggest that certain malignant tumors in man may exert a direct destructive action on the red blood cells *in vitro*. Should a similar phenomenon occur in the living host, a logical explanation of at least one of the causes of anemia in certain cases of cancer would be at hand. Such an explanation would be consistent with reports of recovery from anemia of patients relieved from tumors by surgical procedures.[9] On the other hand it appears difficult to assume that all cases of anemia in cancer are caused by a direct destruction of the red blood cells in the hosts; frank hemolytic anemia has been occasionally observed in neoplastic diseases,[10,11] but is far from being either frequent or usual. It is apparent, therefore, that some other mechanism would have to be also, at least partially, responsible for the development of anemia in certain cases of cancer. Thus, theoretically at least, one could assume that the destructive action of a diffusible substance, either liberated or produced by the tumor, may not be limited to erythrocytes, but may affect, in certain instances, among some other cells, also those of the bone marrow. This, however, is speculation only at the present time.

The fact remains unchanged, nevertheless, that human cancer cells either liberate or secrete a thermolabile substance which, *in vitro* at least, exerts a slow acting, but destructive influence on human erythrocytes.

Summary. Experiments reported in this paper suggest that extracts prepared from human cancer occasionally agglutinate, and frequently hemolyze red blood cells *in vitro*. These phenomena, but particularly the hemolysis, become evident only after a prolonged incubation of the tumor extracts with the red blood cells at 37°C. The agglutination is evident after 24 hours of incubation or earlier; the hemolysis requires 48 hours of incubation. Heating of the tumor extracts for 30 minutes to 56°C, prior to mixing them with the red blood cells, destroys their ability to either agglutinate or hemolyze the erythrocytes.

Mrs. Ruth G. Zahler rendered very ably technical assistance in this study. Dr. B. S. Gordon, Chief of the Clinical Laboratory was responsible for the pathological diagnosis of the slides. Mr. S. Shapiro, Chief of Medical Illustration, was responsible for the photographs.

6 Gross, L., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 341.

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17026

Cell Proliferation Accelerating and Inhibiting Substances in Blood Serum During Pregnancy.*

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In blood serum there are two types of factors which affect cell proliferation in bone marrow cell and tissue cell cultures *in vitro*.[1] Normal blood serum will accelerate the rate of cell proliferation in bone marrow cultures because of a predominance of accelerating over inhibiting factors. Serum from cases of neoplastic disease, pernicious anemia, aplastic anemia and leukemia have a ratio of factors such that a supplement of the serum will inhibit normal cell proliferation. In a series of conditions previously reported,[2] the sera, from 7 cases of pregnancy, inhibited the rate of cell proliferation.

A further study was made of the effect of blood serum from cases of pregnancy on cell proliferation in bone marrow cultures *in vitro*. The technique used for the culturing of bone marrow cells was the same as that previously described.[2] Rabbit bone marrow was used in the experiments reported in this paper.

Fig. 1 gives the response of the cells of bone marrow cultures *in vitro* to the addition of human pregnancy blood serum at various stages of pregnancy. Circles connected by a line represent cases in which three or more specimens of blood serum were obtained at the intervals indicated. Determinations have been made on thirty cases in addition to those shown in Fig. 1, especially in the latter stages of pregnancy. The results all fall close to the order of magnitude of values given in the curves shown and are omitted to prevent overcrowding of the graph. During pregnancy there was an increase in factors which inhibit normal cell proliferation, with a tendency toward an increasing inhibition with the progress of pregnancy. In the later stages of pregnancy, the blood sera were strongly inhibitory of normal cell proliferation similar to the blood sera from cases of neoplastic disease, pernicious and aplastic anemia, and leukemia. The anemia associated with pregnancy is consistent with the change in normal cell accelerating and inhibiting factors as is also the anemia associated with neoplastic disease.

Fig. 2 gives the response of bone marrow cultures *in vitro* to the blood serum of pregnant rats at various stages of pregnancy. The blood serum showed a progressive increase in inhibiting factors up to the time of parturition. The amniotic fluid was obtained from the uterus of the pregnant rats on approximately the eleventh and fifteenth day of pregnancy and at the time of birth. The amniotic fluid becomes strongly inhibitory during the pregnancy, but at the time of birth there was a considerable excess of normal cell, proliferation accelerating factors in the fluid.

At various stages of pregnancy, the rats were sacrificed and the cells of the embryos or fetuses cultured by a technique similar to that used for culturing normal tissue cells and cancer cells *in vitro*.[3,4] The entire embryos were disintegrated in a modified Waring blender as previously described for the preparation of tissue cell suspensions with the addition of Tyrode's solution without glucose. The cell suspension was incubated in 5 ml rubber capped vials at 37°C. Ten mg of acid hydrolyzed casein per ml of cell suspension was added as a neutralized solution of commercial casein hydrolysate. One half mg of

* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

1 Norris, E. R., and Majnarich, J. J., Proc. Soc. Exp. Biol. and Med., 1949, **70**, 229.

2 Norris, E. R., and Majnarich, J. J., *Am. J. Physiol.*, 1948, **153**, 483.

3 Norris, E. R., and Majnarich, J. J., *Am. J. Physiol.*, 1948, **153**, 488.

4 Norris, E. R., and Majnarich, J. J., *Am. J. Physiol.*, 1948, **153**, 492.

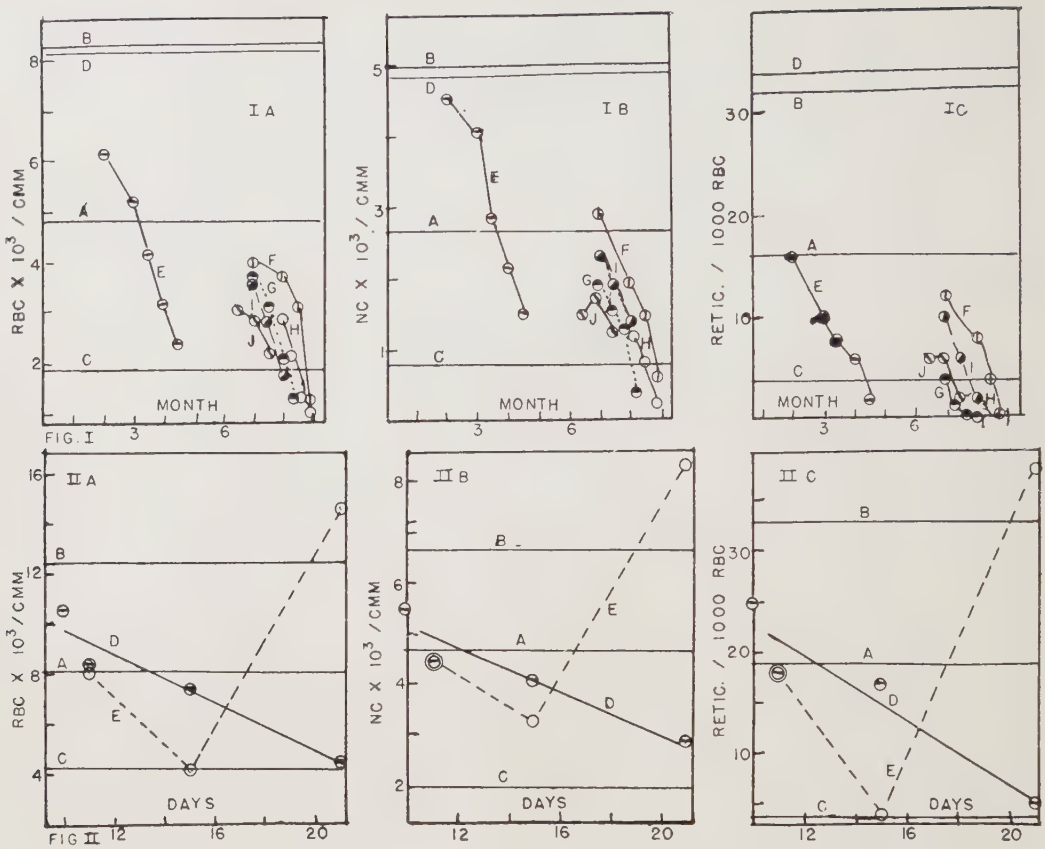

FIG. 1.

The effect of blood serum from cases of human pregnancy at different periods of pregnancy upon the rate of cell proliferation in bone marrow cell cultures *in vitro*. The initial concentration of cells in the bone marrow cell suspension used was red blood cells (RBC) 4840/emm; nucleated cells (NC) 3240/cmm; reticulocytes 10/1000 RBC. Time of incubation of bone marrow cultures is 8 hours. A, indicates the level of response obtained without supplement; B, indicates the level of response obtained with 5 γ of xanthopterin per ml of suspension; C, indicates the level of response with 5 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml of suspension; D, the level of response with normal human serum; E, F, G, H, I, J, the response obtained with serum from cases of pregnancy at the period of pregnancy indicated. One tenth ml of serum was added to 2 ml of bone marrow suspension.

FIG. 2.

The effect of the blood serum and amniotic fluid of pregnant rats upon cell proliferation in bone marrow cultures *in vitro*, at various stages of pregnancy. A, is the level of response obtained with no supplement; B, is the level of response obtained with a supplement of 5 γ of xanthopterin per ml of bone marrow suspension; C, is the level of response obtained with 5 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml of bone marrow suspension; D, is the response obtained with 0.1 ml of blood serum from rats, at different stages of pregnancy, added to 2 ml of bone marrow suspension; E, is the response obtained with 0.1 ml of amniotic fluid from the uterus of pregnant rats, at different stages of pregnancy, added to 2 ml of bone marrow suspension.

tryptophan was added per ml of cell suspension, and supplements as indicated below.

In the earliest stages of pregnancy, it was not possible to separate the implanted embryos from the uterus so that the entire uterus containing the embryos was disintegrated and a cell suspension obtained. Fig. 3 gives response obtained with cells of the uterus from a rat in the early stages of pregnancy compared with that of a suspension of

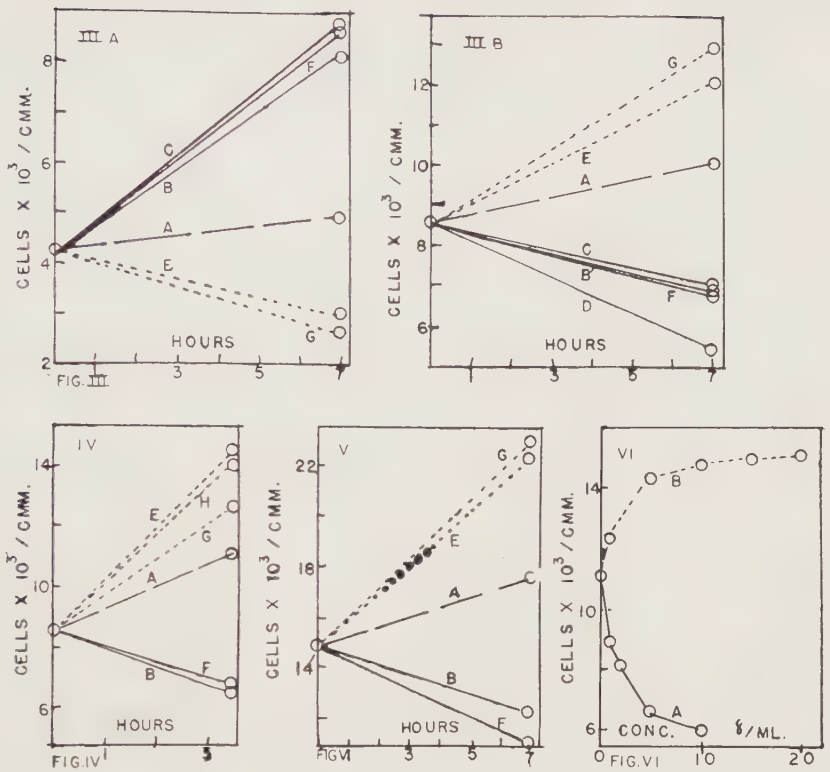

FIG. 3.

The effect of various supplements upon the rate of cell proliferation *in vitro* in a suspension of cells from the uterus of a non-pregnant rat (III A) and from the uterus and embryos of a obtained with 0.1 ml of blood serum from rats, at different stages of pregnancy, added to 2 ml of cell suspension: A, no supplement; B, 5 γ of xanthopterin per ml; C, 1×10^{-6} γ of Vitamin B_{14} per ml; D, 1×10^{-2} γ of Vitamin B_{14} per ml; E, 5 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml; F, 0.1 ml of normal human blood serum; G, 0.1 ml of blood serum from a person with cancer.

FIG. 4.

The effect of various supplements upon the rate of cell proliferation *in vitro* in a suspension of cells, from rat fetuses of about 10 days. The following supplements were added to 2 ml of cell suspension: A, no supplement; B, 5 γ of xanthopterin per ml; E, 5 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml; F, 0.1 ml of normal human blood serum; G, 0.1 ml of human cancer blood serum; H, 0.1 ml of human leukemia blood serum.

FIG. 5.

The effect of various supplements upon the rate of cell proliferation *in vitro* in a suspension of cells from rat fetuses of about 15 days. The following supplements were added to 2 ml of cell suspension: A, no supplement; B, 5 γ per ml of xanthopterin; E, 5 γ per ml of 2-amino-4-hydroxy-7-methyl pteridine; F, 0.1 ml of normal human blood serum; G, 0.1 ml of human cancer blood serum.

FIG. 6.

The effect of various concentrations of pteridines upon the rate of cell proliferation *in vitro* in a suspension of cells from rat fetuses of about 10 days. A, xanthopterin; B, 2-amino-4-hydroxy-7-methyl pteridine.

cells produced from the uterus of a non-pregnant rat. The non-pregnant uterus gave the response obtained with normal tissue cells *in vitro*. The response of the uterus containing the implanted embryos at an early stage gave the opposite response to that of normal cell proliferation. Xanthopterin, Vitamin B_{14}[5]

[5] Norris, E. R., and Majnarich, J. J., *Science*, in press.

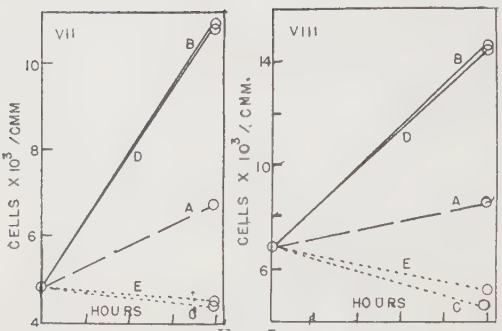

FIG. 7.

The effect of various supplements upon the rate of cell proliferation *in vitro* in a suspension of cells from rat fetuses taken at the time of birth. The following supplements were added to 2 ml of cell suspension: A, no supplement; B, 5 γ of xanthopterin per ml; C, 5 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml; D, 0.1 ml of normal human blood serum; E, 0.1 ml of human cancer blood serum.

FIG. 8.

The effect of various supplements upon the rate of cell proliferation *in vitro* in a suspension of cells from rat young about 12 hours after birth. The following supplements were added to 2 ml of cell suspension: A, no supplement; B, 5 γ of xanthopterin per ml; C, 5 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml; D, 0.1 ml of normal human blood serum; E, 0.1 ml of human cancer blood serum.

and normal blood serum accelerated the rate of cell proliferation of the non-pregnant uterus and inhibited proliferation of the cells of the pregnant uterus. Two-amino-4-hydroxy-7-methyl pteridine and cancer serum inhibited cell proliferation in the non-pregnant uterus cell suspension and accelerated the rate of cell proliferation in the pregnant uterus cell suspension.

Fig. 4, 5 and 6 give the response of cell suspensions obtained from fetuses on approximately the 10th and 15th day of pregnancy. The response was the same as that obtained with the uterus in the early stages of pregnancy described above and also the same as that obtained with neoplastic tissue cell suspensions.[4]

Fig. 7 and 8 give the response of cell suspensions obtained from the fetuses or young at the time of, and soon after birth. The response is that of normal cell suspensions. At some time between approximately the 15th day of pregnancy and the 21st day or the time of parturition, there was a complete change in the response of the cell proliferation to pteridines. In the early embryos and the fetuses up to at least the 15th day, the proliferation of the cells obtained as a suspension was inhibited by xanthopterin and accelerated by 2-amino-4-hydroxy-7-methyl pteridine. At the time of birth the rate of cell proliferation of the cells obtained from the young was accelerated by xanthopterin and inhibited by 2-amino-4-hydroxy-7-methyl pteridine.

Summary. 1. Two types of factors which affect cell proliferation have been observed in blood serum, one of which accelerates the rate of normal cell proliferation, and the other inhibits the proliferation of normal cells. Normal blood serum contains a predominance of factors which accelerate the rate of normal cell proliferation. During pregnancy there is a progressive change in the balance of factors which affect cell proliferation such that the factors which inhibit proliferation of normal cells become predominant.

2. The rate of cell proliferation of a cell suspension *in vitro* of cells obtained from rat embryos and fetuses, at least up to the 15th day of pregnancy, is accelerated by 2-amino-4-hydroxy-7-methyl pteridine and cancer blood serum and inhibited by xanthopterin and normal human blood serum.

3. The rate of cell proliferation of a cell suspension *in vitro,* of cells obtained from rat young at the time of birth, is accelerated by xanthopterin and normal human blood serum and inhibited by 2-amino-4-hydroxy-7-methyl pteridine and cancer blood serum.

17027

Spectrophotometric Method for Assay of Serum Antiprotease: Clinical Applications.*

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(Introduced by S. C. Harvey.)
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Variation in the level of antiproteolytic activity in the serum has been studied in some detail since the presence of this factor was first reported in 1893.[1] The methods for determination of this activity in the past have largely been antifibrinolytic in nature,[2-5] and are not entirely satisfactory because of the subjectivity of the determinations and the variations in result due to differences in substrate and slight variations in handling of the tubes containing clots. A simpler and more objective procedure was described recently from this laboratory.[6] This was still time-consuming, and a more rapid, objective method of assay, to be described, was developed. The results obtained on examination of 250 sera by this method and by the previously reported clot formation method were collected and a statistical analysis was made of the correlation between the two tests.

Materials. 1. Trypsin‖ solution 70 mg% dissolved in veronal buffer, shaken well and filtered, kept cold until used and prepared daily.

2. Fibrinogen¶ solution, 0.5% in veronal buffer.

3. Trichloracetic acid (USP), 16% solution.

4. Sodium hydroxide (reagent grade), 1 N.

5. Saline solution, 0.9%.

6. Veronal buffer,[6] pH 7.4.

7. Folin-Ciocalteau phenol reagent,†† diluted 1:3 with distilled water.

Method. Blood is drawn into dry centrifuge tubes using a dry syringe and needle. Serum is obtained by centrifugation. The serum is diluted to 1:40 with saline, and 0.5 ml of the serum dilution is transferred to a 13 x 100 mm pyrex tube. Two similar tubes for uninhibited trypsin are set up with 0.5 ml of saline. A blank is prepared with 1.5 ml veronal buffer and is carried through the routine, except that trypsin is not added.

All tubes, with the blank tube first, are placed in an ice water bath. When temperature equilibrium has been reached (10 minutes), 1 cc of trypsin solution is added to each tube, the tubes are shaken, and returned to the bath at 30 second intervals (in order). After 30 minutes, in the same order and with the same time interval, 2 ml of fibrinogen are added to each tube. After the addition, each tube is shaken slightly and placed in a water bath at 37°C for 30 minutes.

At the end of this time 5.0 ml of trichlor-

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§ Fellow of the Department of Surgery (Oncology), Yale University School of Medicine.

1 Hildebrandt, H., *Virchows Arch. f. Path. Anat.*, 1893, **131**, 5.

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4 Jobling, J. W., Peterson, W. F., and Egstein, A. A., *J. Lab. and Clin. Med.*, 1915, **1**, 172.

5 Grob, D. (a) *J. Gen. Physiol.*, 1942, **26**, 405; (b) *J. Gen. Physiol.*, 1942, **26**, 423.

6 Clark, D. G. C., Cliffton, E. E., and Newton, F. L., Proc. Soc. Exp. Biol. and Med., 1948, **69**, 276.

‖ Armour trypsin powder.

¶ Armour bovine fibrinogen; Fraction I from bovine plasma.

†† Hartman-Leddon Company.

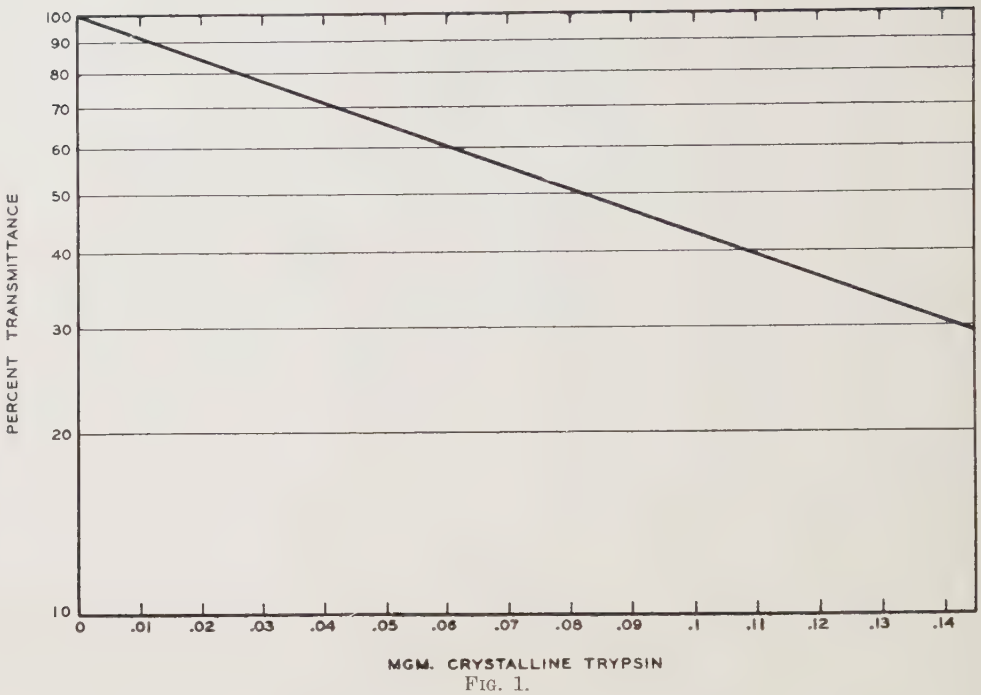

FIG. 1.

acetic acid are added to each tube without shaking and they are left at room temperature for 15 minutes.

The contents of each tube are then filtered through Whatman No. 3 filter paper. When filtration has been completed, a 2.5 ml aliquot of each filtrate is added to 5 ml of NaOH in a 50 ml Erlenmeyer flask. 1.5 ml of diluted phenol reagent is then added at definite time intervals and the contents of the flask are transferred to a standard cuvette. Ten minutes are allowed for color development, when the samples are read at the same time interval against the fibrinogen blank set at 100% transmittance as a reference. A Coleman Spectrophotometer Model 14, set at a wave length of 675 mμ, was employed.

Calculations. The % T (transmittance) readings of uninhibited trypsin and each serum dilution are converted to crystalline trypsin equivalents (CTE) by use of a conversion graph (Fig. 1). The equivalent number of milligrams of crystalline trypsin inhibited by each serum sample is then determined by subtraction of its CTE from the average CTE of the 2 uninhibited trypsins. Tests performed over a period of four weeks have shown that serum of the average patient with no apparent disease inhibits 0.0167 mg of crystalline trypsin. This figure may be used for statistical purposes; however, it is not dependable for individual daily tests because of variations in the reagents, which are prepared daily. Any daily variations are proportional for normal individuals and for those with malignant neoplasia or other diseases. For this reason at least one normal control serum is analyzed with each daily group and the unknowns are reported as % of the normal arbitrarily fixed at 100%.

Results. Tests were performed on 250 samples of blood from patients with various diseases and control patients without evident disease. The results are essentially the same with the two methods (clot formation and spectrophotometric). Although individual cases do not coincide exactly, definite differences occur but rarely. A statistical analysis**

** The statistical analysis was made by Mrs. Sylvia Johnson of the Department of Public Health and the Department of Surgery (Oncology), Yale University School of Medicine, assisted by Mr. David Votaw of the Department of Mathematics, Yale University.

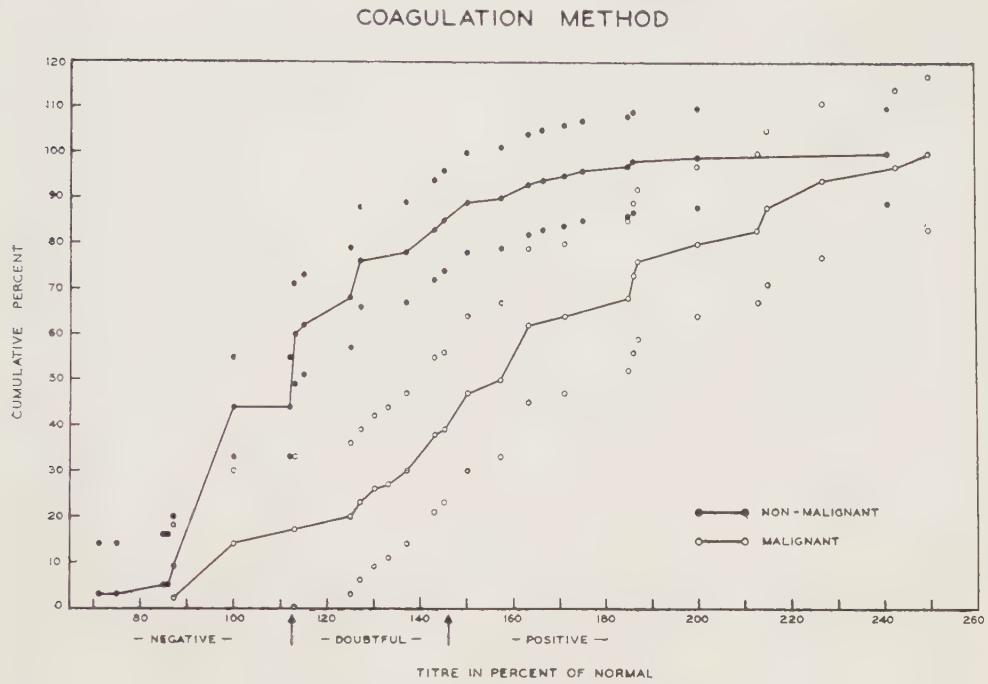

FIG. 2.

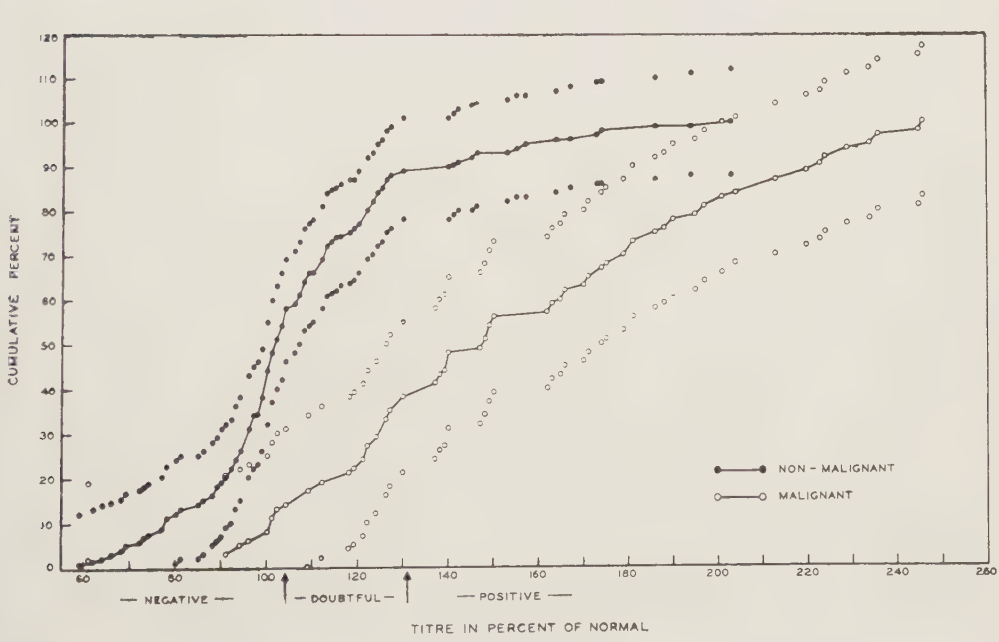

FIG. 3.

of the results in relation to the presence or absence of malignant tumor in the patients studied revealed that the results are comparable (Fig. 2 and 3). This analysis of these cases revealed that using a normal control at 100%, a titre below 112.5% for the clot formation method and below 103% for the spectrophotometric method could be consid-

ered negative with a possible error of 5%, and that a titre above 146% for the clot formation method and above 130% for the spectrophotometric method could be considered as indicative of malignant neoplasia with a possible error of 5%. With the clot formation method, titres between 112.5% and 146% and with the spectrophotometric method titres between 103% and 130% must be considered of doubtful significance.

These results indicate that this spectrophotometric method may be as satisfactory for the detection of patients with malignant neoplasia as the previously reported method.

False positive results with the spectrophotometric method fall in the same groups as with the clot formation method, *i.e.* those patients with acute infectious diseases, especially streptococcal infections, those with advanced tuberculosis of the lungs and those recently subjected to major operations.

Summary. A spectrophotometric method of assay of antiproteolytic material in the serum, using inhibition of trypsin digestion of fibrinogen, has been described. Results obtained in 250 sera by this method have been analyzed statistically and have been found to show a close correlation with the clot formation method.

17028

Microphonic Manometer for Indirect Determination of Systolic Blood Pressure in the Rat.*

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Although the plethysmographic method for the indirect measurement of systolic pressure in the rat[1] has been improved by modifications devised later by other workers,[2-5] it has not been adopted universally. This has been due primarily to technical difficulties and errors encountered in any method employing plethysmographic changes for determination of blood pressure.

We have devised a method, however, of obtaining the systolic blood pressure of the rat which is similar in principle to that employed in the indirect measurement of systolic blood pressure in man,—namely, the detection of sound at the exact time that the arterial pressure of the caudal artery exceeds the pressure of the occluding cuff. This method was found to be accurate, simple, and rapid.

The apparatus consists essentially of a carbon type of microphone (3 cm in diameter) to the diaphragm of which is attached a thin copper trough (1.5 cm in length and 0.6 cm in diameter). The microphone is connected to a specially designed, low frequency (100 c.p.s.) sound amplifier† operated by direct current supplied by three dry cells. This amplifier affords a voltage gain of approximately 3000. The energy changes obtained by the amplifier may be detected by ordinary earphones or oscilloscope.

In order to take blood pressure readings, a

* Aided by a grant from the United States Public Health Service.

1 Williams, J. R., Jr., Harrison, T. R., and Grollman A., *J. Clin. Invest.*, 1939, **18**, 373.

2 Kempf, G. F., and Page, I. H., *J. Lab. and Clin. Med.*, 1942, **27**, 1192.

3 Proskauer, G. G., Neumann, C., and Graef, I., *Am. J. Physiol.*, 1945, **143**, 290.

4 Sobin, S. S., *Am. J. Physiol.*, 1946, **146**, 179.

5 Skeggs, L. T., Jr., and Leonards, J. R., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 294.

† Further details of the construction of this apparatus and the electrical circuit will be furnished by the authors upon request. The microphonic manometer itself may be obtained from Charles Calvert of the Pacific Industrial Electronics Company, San Francisco, Calif.

rat is placed for 10 minutes in a wood box (55 x 25 x 25 cm) having sliding doors at each end and a glass top. The temperature of the box is maintained at approximately 39°C. The tail of the rat is led through a 10 mm pressure cuff and the segment of the tail immediately distal to the cuff is placed in the trough. The pressure of the cuff is raised to a point above the expected blood pressure and slowly released. At the point at which the cuff pressure becomes less than the caudal arterial pressure, one *immediately* hears in the ear phones a rhythmical succession of sounds reflecting at exactly the same rate the transmitted pulsatile variations of the caudal artery. If the amplifier is connected to an oscilloscope, one sees at this same critical point an intense and abrupt change in the contour and frequency of the preceding waves. It should be stressed that the regularity and actual rate of these sounds do not allow their confusion with any possible extraneous movements of either the body or tail of the rat. As the cuff is deflated further, the sounds attain their greatest intensity at the expected diastolic level, although this increase in intensity is too gradual to allow precise determination of the diastolic pressure. The sounds continue even with the cuff completely deflated. No preliminary venous drainage of the tail is necessary as the method does not depend upon gradual increase in tail volume but upon the almost instantaneously transmitted pulsatile variations which occur when the cuff pressure is overcome. Blood pressure determinations can be made at intervals of 15 to 30 seconds.

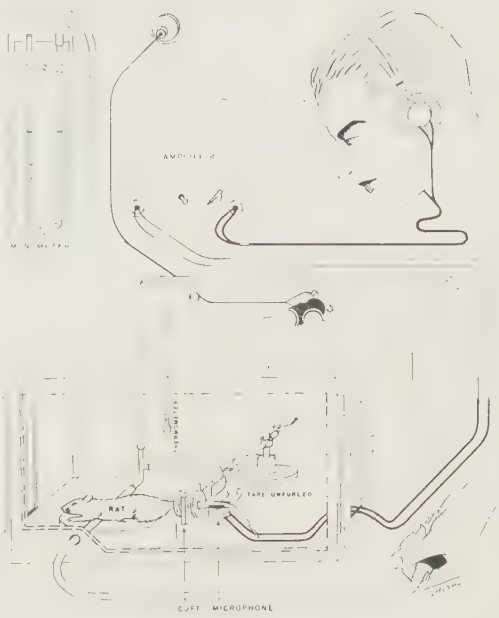

FIG. 1.

Drawing depicts entire apparatus. The rat is placed on a pad of sponge rubber. The cuff is inflated by hand bulb and air pressure is measured by manometer. As the air is released, the cuff pressure falls below that in tail artery and the pulsatile vibration instantly detected by the microphone is amplified into rhythmic audible signals heard by the observer.

Results. A. *Blood Pressure of the Normal Rat* (1) *Unanesthetized Rats.* Twelve albino rats weighing between 150-200 g were given 0.02 mg of Intracostrin (Squibb) per 100 g of body weight. This amount of curare, although insufficient to produce paralysis, did subdue the rats sufficiently that they remained relatively quiet when wrapped loosely in a cloth binder. The average systolic blood pressure of these rats as determined by the microphonic manometer was 98 mm of Hg. (Range: 82 to 120 mm Hg). Repeated daily determinations of the same animal rarely deviated more than 4-10 mm of Hg from the initial reading. These determinations are comparable to those obtained by previous investigators.[1,2]

(2) *Anesthetized Rats.* Twenty rats similar to those above were anesthetized by intraperitoneal injection of pentobarbital sodium. The average systolic blood pressure of these rats was 90 mm of Hg (Range: 70 to 104 mm of Hg). Later subsequent determinations of the same animals did not deviate more than 4-8 mm of Hg from the initial value.

B. *Blood Pressure of Rat after Injection of Epinephrine and Renin.* Five anesthetized rats whose average initial pressure was 91 mm of Hg (Range: 82 to 102 mm of Hg) were given 0.2 mg of epinephrine in oil by subcutaneous injection. Thirty minutes later, the average pressure was 180 mm of Hg (Range: 144 to 208 mm of Hg).

Twelve anesthetized rats were given one mg of purified hog renin by intravenous injection. The pressure of each rat was de-

termined immediately and continuously after the injection. It was observed that the average pressure of 88 mm of Hg before injection increased to a maximal average of 170 mm of Hg approximately two minutes after injection. Similar to the pressor response of the dog to renin, the pressures of the injected rats gradually fell after maintaining a plateau for approximately five minutes after injection. The pressures usually reached the control levels about 20 minutes after injection. Again similar to the dog, the rat was observed to develop tachyphylaxis rather quickly to injections of renin. Thus successive injections of the same amount of renin effected progressively smaller and more evanescent pressor effects and the third or fourth successive injection usually caused little or no rise in pressure.

Summary. The ability of the microphonic manometer to measure rapidly and simply not only the pressure of the intact rat but also of the rat made hypertensive by the injection of the vasoconstrictor substances, epinephrine and renin, indicates that it might be of considerable usefulness in studies necessitating frequent or successive blood pressure determinations in the rat.

The authors would like to express their appreciation for technical assistance rendered by Vivian Seay.

17029

Kinetics of Distribution of Inulin Between Two Body Water Compartments.

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The kinetics of distribution of inulin between the intravascular and the interstitial compartments after its introduction into the circulatory system is of interest in relation to the use of this substance as a measure of the extracellular volume.[1,2]

The present paper concerns the distribution of any substance with the properties of inulin between two fluid systems representing schematically the intravascular and interstitial compartments of the body.

Let V_1 be the volume† of the intravascular compartment (I) and V_2 the volume of the interstitial compartment (E), both of which will be assumed to remain constant during the experimental procedure. It is further supposed that the rate of mixing of the substance in each of these compartments is rapid compared to simple diffusion. If the concentration of the substance in I is represented by $c_1(t)$, the concentration in E by $c_2(t)$, the concentration in the urine (assumed to be the only route of elimination) by $c_3(t)$, and if the flow (q_1) of the liquid that is being interchanged between the two compartments is assumed to be constant, q_2 being the urine flow, then, $c_1(t)V_1$ is the amount of substance accumulated in I at any instant, $c_2(t)V_2$ the amount accumulated at any instant in E, $[c_1(t)—c_2(t)]\ q_1$ the rate of interchange of the substance between I and E, and finally, $c_3(t)\ q_2$ is the rate of elimination. In the case under consideration, the substance (inulin) is eliminated only by glomerular filtration, and if the renal clearance at any instant is q_3, then $c_3(t)\ q_2 = c_1(t)\ q_3$. The substance will be introduced into the system in the form of a chemical solution with a rate CQ, where C is its concentration and Q is the rate of infusion, both C and Q being kept constant experimentally.

Under these conditions, the rate of accumulation of the substance in the vascular compartment will be:

$$V_1 \frac{dc_1}{dt} = QC - q_1(c_1 - c_2) - q_3c_1 \qquad (1)$$

* Dazian Foundation Fellow.

1 Gaudino, M., Schwartz, I. L., Levitt, M. F., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 507.

2 Gaudino, M., Levitt, M. F., *Am. J. Physiol.*, 1949, in press.

† Volumes are expressed in cc of fluid, independently of any volume effects of protein, flows in cc per minute, concentrations in mg per cc.

and the rate of accumulation in the interstitial compartment:

$$V_2 \frac{dc_2}{dt} = q_1(c_1 - c_2) \qquad (2)$$

Equation (1) and (2) form a system of linear differential equations the solutions of which are:

$$c_1(t) = A\, e^{\lambda t} \qquad (3)$$

$$c_2(t) = B\, e^{\lambda t} \qquad (4)$$

Substituting (3) and (4) into (1) and (2), after appropriate differentiation:

$$V_1V_2\lambda^2 + (V_1q_1 + V_2q_1 + V_2Q_3)\lambda + q_1q_3 = 0 \qquad (5)$$

(5) being the characteristic equation corresponding to the system (1) (2). The roots λ_1 and λ_2 of (5) will be:

$$-(\lambda_1,\lambda_2) = (\beta,\alpha) \qquad (6)$$

and

$$(\beta,\alpha) = \frac{1}{2V_1V_2}\left[(V_1q_1 + V_2q_1 + V_2q_3) \pm \sqrt{(V_1q_1 + V_2q_1 + V_2q_3)^2 - 4q_1q_3V_1V_2} \right] \qquad (7)$$

Depending on the manner in which the substance is introduced in the organism, different solutions of this system of differential equations will be obtained. Three cases will be considered: (a) the substance will be introduced in the form of a rapid and single injection; (b) it will be introduced at a constant rate until uniform distribution between the intravascular and interstitial compartments has been reached; (c) the behavior of the substance will be studied after the cessation of the infusion required to maintain the steady state represented by (b).

(a) *Single injection.* In the case in which a single injection is given (50 cc in about 1 minute) the substance can be assumed to be introduced instantaneously into the system. Then, the following solution holds:

$$c_1(t) = A\, e^{-\alpha t} + B\, e^{-\beta t} \qquad (8)$$

where A and B are integration constants which are calculated from (1) and (8). Their values are:

$$A = \frac{\beta c_1(o)}{\beta - \alpha} \qquad (9)$$

$$B = -\left(\frac{\alpha c_1(o)}{\beta - \alpha}\right) \qquad (10)$$

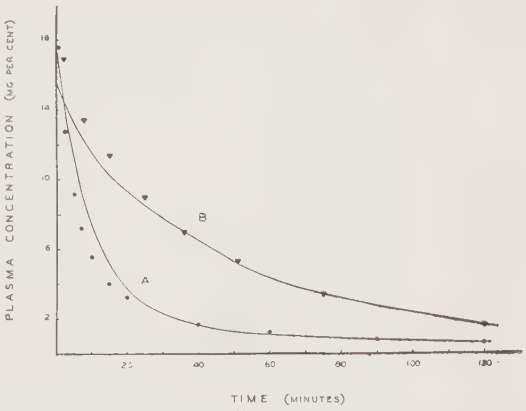

FIG. 1.

Rate of disappearance of inulin from the plasma after a single injection (A) and after a constant infusion (B). The curves are calculated from equations (8) and (17) respectively, the values $\alpha = 0.01$, $\beta = 0.10$, A = 2.0, B = 15.2, P = 12.4, R = 3.0, being obtained by trial and error. The two curves were obtained in the same dog.

$c_1(o)$ representing the concentration of the substance in I at $t = 0$.

Equation (8), with the appropriate substitutions, gives the solution of this case and permits the calculation of the rate of disappearance of the substance from the intravascular compartment.

To test this equation, single injections of inulin were given to 3 normal dogs and the plasma concentration was determined at frequent intervals and related to time. The results were identical in all of the experiments, the observed curve corresponding closely with the theoretical curve calculated by giving values to the constants in (8), (Fig. 1A).

(b) *Constant infusion.* If the rate of introduction of the substance is the same as the rate of elimination (a condition attained by constant intravenous infusion), then,

$$q_3c_1 = QC \text{ or } c_1 - \frac{QC}{q_3} = 0 \qquad (11)$$

Letting

$$c_1 - \frac{QC}{q_3} = y, \qquad (12)$$

the general solution of (5) is

$$y = K\, e^{-\alpha t} + D\, e^{-\beta t} \qquad (13)$$

and hence

$$c_1(t) = \frac{QC}{q_3} + K\, e^{-\alpha t} + D\, e^{-\beta t} \qquad (14)$$

in which α and β are defined by (7) and K and D are constants of integration with the following values:

$$K = \frac{QC - V_1\beta\left(\frac{QC}{q_3}\right)}{V_1(\beta - \alpha)} \quad (15)$$

$$D = -\left[\frac{QC - V_1\alpha\left(\frac{QC}{q_3}\right)}{V_1(\beta - \alpha)}\right] \quad (16)$$

that can be replaced in (14).

According to equation (14) when t becomes infinite the concentration of the substance in the blood will depend only on the rate of infusion and on the rate of glomerular filtration. As both are supposed to be constant, the plasma concentration also becomes constant. Further, since it was assumed that the rate of mixing of the substance in both compartments is rapid, the concentration in the interstitial space can be considered the same as the concentration in the plasma water.

Experimentally, this equilibrium has been attained in 2 hours in the dog.[2] In both cases the blood level follows the general pattern described by (17).

(c) *After cessation of constant infusion.* The general solution of (5) after a constant infusion has been discontinued is:

$$c_1(t) = P\,e^{-\alpha t} + R\,e^{-\beta t} \quad (17)$$

where P and R are integration constants with the values:

$$P = \frac{\beta V_1 c_1(n) - q_3 c_1(n)}{V_1(\beta - \alpha)} \quad (18)$$

$$R = -\left(\frac{\alpha V_1 c_1(n) - q_3 c_1(n)}{V_1(\beta - \alpha)}\right) \quad (19)$$

Here $c_1(n)$ is the concentration in the plasma water at the moment of interrupting the infusion when $t = n = O$. It will be observed that the only difference between (8) and (17) lies in the values of the integration constants.

The decrement in plasma concentration in relation to time was studied in 3 normal dogs after the interruption of a constant infusion of 2 hours duration. The results obtained were similar in the three cases and again show good correspondence with the theoretical curve obtained by giving values to P and R in (17) (Fig. 1B).

The difference between the curves obtained (a) after a single injection and (b) after constant infusion to equilibrium shows that uniform distribution is never reached after a single injection.

Further, as during constant infusion, the condition stated in (11), namely that $CQ = q_3c_1$, is fulfilled at the moment of interruption the amount of substance (Z) contained in both I and E will disappear from the body at a rate expressed only by q_3c_1. The total amount of inulin excretion will then be given by:

$$Z = q_3\int_0^\infty c_1(t)\,dt \quad (20)$$

Introducing (17) with the appropriate substitution in (20), integrating within the limits indicated, and substituting the values of α and β from (7), the result is:

$$(V_1 + V_2) = \frac{Z}{c_1(n)} \quad (21)$$

where $V_1 + V_2$ is the total volume of the extracellular space. This volume can then be calculated by dividing the total amount (Z) of inulin excreted since the interruption of the infusion, by the concentration $c_1(n)$ of this substance in the blood at the moment of interruption, as previously reported.[1,2] The volume of distribution of inulin thus measured corresponds to 19 per cent of the body weight in the dog[2] and to 16 per cent in man.[1]

Summary. Comparison of the rate of disappearance of inulin from the plasma after a single injection and after prolonged constant infusion yields data which, on mathematical analysis, conform with the assumption that in the latter circumstance, inulin is uniformly distributed throughout some fixed volume of body fluid, presumably the extracellular fluid.

The author is deeply indebted to Dr. Domingo M. Gomez for his valuable advice in the consideration of this problem.

17030

Biological Oxidation of Phospholipids by Rat Liver Homogenates.*

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The normal saturated C_4 to C_{18} fatty acids have been shown to be oxidized by homogenized rat liver preparations in the presence of adenosine triphosphate (ATP) and cytochrome c,[1] with acetoacetate as a product of the reaction.[2] Presumably this reaction represents fatty acid oxidation *in vivo*, but the concentration of free fatty acids in animal tissues is actually very low.[3] Hence other lipids may also serve as the source of fatty acid for oxidation, and among these the phospholipids have been strongly implicated as intermediates in fat metabolism.[4] Consequently, in the work reported here, measurements were made of the oxygen consumption and acetoacetate production from phospholipids, utilizing the type of enzyme preparation which oxidizes free fatty acids.

Experimental. Substrates. The petroleum ether-soluble, acetone-insoluble fraction of beef brain lipid was prepared and found to contain 3.3% phosphorus and to have an iodine number of 85.5. It was stored in petroleum ether, under nitrogen, at 5°. The beef lung hydrolecithin was generously supplied by Dr. S. J. Thannhauser. Emulsions of these compounds were prepared by suspending 80 mg of the phospholipid in 10 ml of 0.1 M phosphate buffer, pH 8.0, and homogenizing the mixture in a Potter-Elvehjem glass homogenizer. A 0.01 M sodium octanoate solution, used to test the activity of the enzyme preparations for fatty acid oxidation, was prepared by neutralizing redistilled *n*-octanoic acid with the required amount of NaOH and adjusting the pH to 7.0-7.5.

Reagents. Cytochrome c was prepared from beef heart according to the method of Keilin and Hartree.[5] The stock solutions used varied from 1.3 to 2.0 $\times$ 10^{-4} M. Adenosine triphosphate was prepared from rabbit muscle according to the method of Dounce *et al.*[6]

Analytical methods. For the measurement of oxygen consumption, Warburg flasks of about 20 ml total volume were used. The flasks were constructed with two side arms. All experiments were run at 30° and in all cases the gas phase was air. The center well of each flask was equipped with a filter paper roll and 0.2 ml of 20 per cent KOH. Acetoacetic acid was determined by distilling a metaphosphoric acid filtrate of the incubated samples and determining the acetone by the vanillin method of Alyea and Backström.[7] The detailed procedure has been described by Witter and Stotz.[8]

Liver Homogenate. White rats, which had been starved overnight, were decapitated and bled. The liver was removed and homogenized in the manner described by Lehninger,[1] using an all-glass homogenizer which has been described by Dounce.[9]

The Oxidation of Beef Brain Phospholipid.

* This research was supported by grants from the Nutrition Foundation, Inc., The Sugar Foundation, Inc., and the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

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TABLE I.
Oxidation of Beef Brain Phospholipid.

Substrate	ATP*	Oxygen consumption, cmm	Acetoacetic acid, μM
Endogenous†	+	24	1.95
Octanoate	+	85	4.50
Phospholipid	+	75	1.80
		104	1.80
Phospholipid	—	0	1.00
Endogenous	+	13.6	1.50
Octanoate	+	110	3.60
Phospholipid	+	109	1.55
,,	—	3.2	0.75
Endogenous	+	8	1.40
Octanoate	+	51	2.25
Phospholipid	+	73.1	1.45
		103	1.50
,,	—	13.5	1.05

* When absent, replaced by equal volume of water in side arm.
† Substrate replaced by equal volume of water.

TABLE II.
Oxidation of Beef Lung Hydrolecithin.

Substrate	ATP	Oxygen consumption, cmm	Acetoacetic acid, μM
Endogenous	+	114	—*
Hydrolecithin	+	182	—
,,	—	27.0	—
Endogenous	+	41.0	1.35
Octanoate	+	165	6.70
Hydrolecithin	+	167	1.40

* Not determined.

The main compartment of each Warburg flask contained 0.4 ml of 0.1 M phosphate buffer, pH 7.7, 0.2 ml of cytochrome c, and 0.1 ml of 0.1 M magnesium chloride. One side arm of each flask contained 0.4 ml of 0.01 M ATP solution, adjusted to pH 7.5. The other side arm contained 0.4 ml of substrate solution. Just before equilibration, 0.5 ml of liver homogenate were added to the main compartment of each flask. The equilibration period was 5 minutes, at which time the taps were closed and the contents of the side arms tipped. The oxygen uptake was nearly linear through a 30 minute period of measurement and the results reported are expressed in terms of a total 30 minute oxygen consumption. After the measurements of the oxygen consumption, the contents of the flasks were analyzed for acetoacetic acid. The results of typical experiments are recorded in Table I.

The homogenized rat liver preparation oxidized both free fatty acids and phospholipid in the presence of ATP. Only the oxidation of the free acid resulted in the production of acetoacetic acid in amounts significantly greater than was produced by preparations to which no substrate was added. The necessity of ATP for phospholipid oxidation and the rate of the oxygen consumption suggested that the oxidation was of a true biological nature, rather than an auto-oxidation.

The Oxidation of Beef Lung Hydrolecithin. The above conclusion was supported by results obtained through the use of beef lung hydrolecithin as a phospholipid substrate. Both the fatty acids of the molecule are palmitic acid.[10] Since auto-oxidation is believed to involve the reaction of oxygen with the unsaturated acids

[10] Thannhauser, S. J., Benotti, J., and Boncoddo, N., *J. Biol. Chem.*, 1946, **166**, 669.

of the phospholipid molecule, hydrolecithin should not be susceptible to auto-oxidation.

The hydrolecithin was used as a substrate with the enzyme preparation previously described. The results of typical experiments are recorded in Table II.

Discussion. Since acetoacetic acid is not metabolized by the homogenized rat liver preparation, and there is no inhibition of acetoacetic acid formation when octanoate and phospholipid are added to the same preparation, the failure of the oxidizing phospholipid to produce acetoacetic acid indicates that free fatty acids are not liberated from the phospholipid in the preparation employed.

It was found that the rat liver homogenate was able to dehydrogenate phospholipid in the presence of ATP, as measured by the Thunberg technic. The aerobic reaction, however, did not result in an increased phospholipid iodine number, and it was found impossible to determine what changes might have occurred in the fatty acids of the phospholipid as a result of the oxidation. Nevertheless, the fact that both free fatty acids and phospholipid are oxidized by the same enzyme preparation prompts the suggestion that this new oxidative reaction of phospholipid is somehow related to intermediary fatty acid metabolism.

Summary. Beef brain phospholipid and beef lung hydrolecithin are oxidized by rat liver homogenates in the presence of adenosine triphosphate. Although this preparation oxidizes free fatty acids with the production of acetoacetic acid, the phospholipid oxidation does not result in such a product.

17031

Effects of Glucose Fermentation Products on Determination of Mannitol by Periodate-Titrametric Method.

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When mannitol clearance and maximal glucose reabsorptive capacity are measured simultaneously in studies of renal function,[1,2] it becomes necessary to determine mannitol in the presence of high concentrations of glucose in both plasma and urine. Satisfactory recoveries of mannitol under these conditions have been reported by others,[2] but certain discrepancies have been noted by us. This report deals with a) the extent of interference of glucose fermentation products with the determination of mannitol, b) a correction factor, and c) the nature of the interfering substance.

Glucose was added to water in varying concentrations from 0 to 1250 mg %. The increases were made in steps of 50 mg %. The solutions were then analyzed for mannitol by the method of Smith, Finkelstein, and Smith[3] except that $CdSO_4$-NaOH[4] was used instead of $ZnSO_4$-NaOH as the precipitating reagent, and the time of fermentation was extended to 2 hours. The method entails fermentation of glucose from the samples by yeast, removal of yeast by centrifugation, precipitation of the proteins, oxidation of the mannitol in the filtrate with periodic acid, and the determination of the excess periodic acid, together with the iodic acid formed, by titration with sodium thiosulfate. All of the filtrates used in our determinations were analyzed quantitatively for glucose[5] to make cer-

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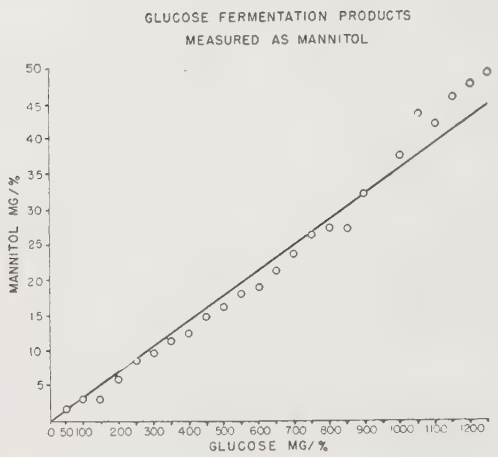

FIG. 1.

tain that the fermentation was complete. The titrations of the filtrates for mannitol were found to increase almost in direct proportion to the quantity of glucose removed. (Fig. 1). An analysis of the results revealed that 3.51% of the glucose fermented was measured as mannitol.

Attempts were made to control the formation of measurable glucose fermentation products by using a constant temperature incubator and increasing the temperature to 37°C. Recovery of apparent mannitol was thus increased to 4.9% of the glucose fermented, indicating that an increased and more variable production of the interfering substance had occurred.

Under optimum conditions for fermentation of glucose by yeast at 25 to 30°C and at a pH 5.6 to 5.8,[6] the glycerol formed is equivalent to 3 to 4% of the glucose destroyed.[7] Periodic acid, which is used for the oxidation of mannitol, will also oxidize the glycerol,[8] each cc of 0.005 N sodium thiosulfate being equivalent to 0.091 mg of mannitol or to 0.093 mg of glycerol. Para-aminohippuric acid is also oxidized by periodic acid, and hence interferes with the determination of mannitol. The method of Barker and Clark[9] prevents this interference, but not the reaction of periodic acid with the fermentation products of glucose.

When the effect of the glucose fermentation products are ignored, the mannitol clearance values determined simultaneously with the maximal glucose reabsorptive capacity are lowered, the degree depending upon the relative amounts of glucose and mannitol in plasma and urine. The amounts vary with the ratio of the mannitol clearance to the maximal tubular glucose reabsorptive capacity, the mannitol clearance value, and the plasma levels of mannitol and glucose. The details of the factors which affect the mannitol clearance are shown in the following equation:

$$\frac{U_M V + ((C_M P_G - T_m G) \times \text{Glucose Correction})}{P_M + (P_G \times \text{Glucose Correction})} = C_M \text{ Uncorrected Values.}$$

C_M = mannitol clearance, cc/min.
U_M = urine mannitol, mg/cc.
V = urine vol., cc/min.
P_M = plasma mannitol, mg/cc.
P_G = plasma glucose, mg/cc.
T_mG = max. glucose reabsorptive capacity, mg/min.

The mannitol clearance values are always lowered as the concentration of glucose in the urine is less than that of plasma due to the tubular reabsorption of glucose.

Summary. 1. Glucose fermentation products interfere with the periodate-titrimetric method of mannitol determination. 2. Failure to correct for this interference lowers the mannitol clearance values.

The advice of Dr. Robert W. Keeton, the technical assistance of Mr. Edward Eckert, and the generous donation of mannitol by Dr. J. Wm. Crosson of Sharp and Dohme, Inc., are gratefully acknowledged.

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17032 P

Serum Level of Protein Bound Radioactive Iodine (I^{131}) in the Diagnosis of Hyperthyroidism.*

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With the technical assistance of Barbara Seamon.
(Introduced by Herrman L. Blumgart.)

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It is generally agreed that the blood level of protein bound ("hormonal") iodine is a measure of thyroid activity; in most patients with thyrotoxicosis the plasma protein bound iodine levels are above 8-10 γ %.[1-4] The chemical determination of plasma protein bound iodine is, however, difficult and laborious and despite its importance is not widely used. Recently, Taurog, Chaikoff, and Entenman[5] have used radioactive iodine (I^{131}) to measure the turnover of plasma protein bound iodine in dogs. It seemed possible that thyroid function might be estimated in man by measuring the concentration of serum protein bound radioactive iodine following an oral dose of I^{131}.

Methods. One hundred and fifty microcuries of I^{131}, carrier free, were administered orally to 20 subjects, aged 24 to 65 years; 16 were female. Ten subjects were thyrotoxic; the other 10 were euthyroid. The thyrotoxic subjects had the characteristic symptoms and signs. The basal metabolic rates ranged from +15 to +60, and averaged +34%. The circulation time, blood cholesterol, the body retention of I^{131} as measured by urinary excretion,[6] and the I^{131} uptake in the thyroid measured by external counts[7] were consistent in each instance with the clinical diagnosis of thyrotoxicosis. The other 10 subjects were euthyroid by these criteria. The basal metabolic rate ranged from —10 to +15 and averaged +2%.

The I^{131} was given 3 hours after a light breakfast. Twenty-four hours later, 10 cc of venous blood was obtained from each subject and the serum separated.

Procedure for determination of protein bound radioactive iodine. A. 1.0 cc serum was pipetted into a previously weighed glass boat measuring 25 mm in diameter and 7 mm deep.

B. The protein bound I^{131} was separated by the method of Chaikoff *et al.*[8] modified as follows:

(1) 1.0 cc serum was pipetted into a small centrifuge tube and 1.0 cc 10% trichloracetic acid (cold) added.

(2) After centrifugation (2500 RPM) for 30 minutes, the supernatant was removed and saved.

(3) The precipitate was washed twice with 2-5 cc cold 5% trichloracetic acid. After each addition of trichloracetic acid and centrifugation, the supernatant fluid was removed. The washings were pooled.

(4) The precipitate was dissolved in 1.0 cc 2 N NaOH and transferred to a weighed glass boat.

C. 1.0 cc of the collected supernatant (inorganic fraction) was adjusted to pH 7.5 and

* This work was carried out under a contract of the Office of Naval Research Atomic Energy Commission and the President and Fellows of Harvard College.

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TABLE I.

M.S., Age 62; Duodenal Ulcer; Euthyroid. Oct. 13, 1948, 10 a.m., 150 μc. I^{131}, carrier free by mouth. Oct. 14, 1948, 10 a.m., 10 cc venous blood drawn.

	cc	Dry wt, mg	Net counts/min., corrected for mass and dilution	Correction for decay, %	Net counts /min./cc
A. Total serum	1.0	110	242	77	315
B. Precipitate	1.0	89	50	77	65
C. Filtrate	1.0	54	185	77	239

Background 15 counts/min.
From nomogram 0.001 microcurie = 450 cts./min.
B. Precipitate (Protein Bound I^{131}) 65 cts./min. = .00015 $\mu c = 15 \times 10^{-5}$ μc.

transferred to a previously weighed glass boat.

One drop 10% gelatin (2-3 mg) was added to each cup and the samples evaporated slowly (37 to 40°C) to dryness. The cups were reweighed and the radiation determined with an end window Geiger-Mueller tube (3.2 mg/cm^2). Corrections for mass absorption, dilution, and decay were made. All determinations were extrapolated to the time the sample was obtained.

Calculations. The total serum activity per cc (Table I) should equal the precipitate (protein bound I^{131}) activity, plus the filtrate (inorganic I^{131}) activity. The conversion to microcuries of net counts per minute per cubic centimeter was made by reference to a nomogram. Under these geometric and physical conditions, .001 microcurie I^{131} gave 450 net counts per minute. The reference standard for I^{131} radiation was Bi 210 (half life 22 years).

The following protocol (Table I) is illustrative.

Results. The results are shown in Fig. 1 and 2. In the hyperthyroid patients the serum protein bound I^{131} ranged from 38 to 146 $\times$ 10^{-5} μc/cc (Fig. 1), averaging 68 $\times$ 10^{-5} μc. In the euthyroid patients the serum protein bound I^{131} ranged from 3 to 28 $\times$ 10^{-5} μc/cc averaging 13 $\times$ 10^{-5} μc. Sixty per cent of the observations in the patients with thyrotoxicosis were above 50 $\times$ 10^{-5} μc, whereas in the euthyroid subjects, 70% were below 20 $\times$ 10^{-5} μc.

There was considerable overlap in the total serum counts (Fig. 2). In the thryotoxic patients the total serum activity ranged from 199 to 659 counts/min./cc and in the euthyroid subjects from 50 to 715 counts/min./cc.

Comment. The tracer technic has been increasingly employed in studies of thyroid function. After an oral dose of I^{131}, urinary excretion accounts for most of the loss from the body;[9-11] the remainder as measured by external counts is mainly in the thyroid gland. A more direct estimation of thyroid function is afforded by the serum level of protein bound radioactive iodine. The higher protein bound I^{131} serum level found in thyrotoxicosis is consistent with increased thyroid activity in these patients. The difference in protein bound radioactive iodine serum levels in euthyroid and thyrotoxic subjects has proved useful diagnostically. Our results indicate, that while the counts/cc of whole serum drawn 24 hours after the oral dose are generally lower in euthyroid subjects than in thyrotoxics, the overlap (Fig. 2) precludes differentiation by the relatively simple determination of total serum activity. This overlap may not be present 72 or 96 hours after a tracer dose and is the subject of further studies.

The factors which influenced our choice of a 24-hour period may be summarized. In rats, Chaikoff, Taurog, and Reinhardt[8] have shown that 24 hours after an injection of I^{131}, approximately 90% of the plasma radioactivity is in protein bound form. Preliminary studies[12] on serum obtained from thyrotoxic patients 1, 3, 6, 24, 48 and 72 hours

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[12] Unpublished data.

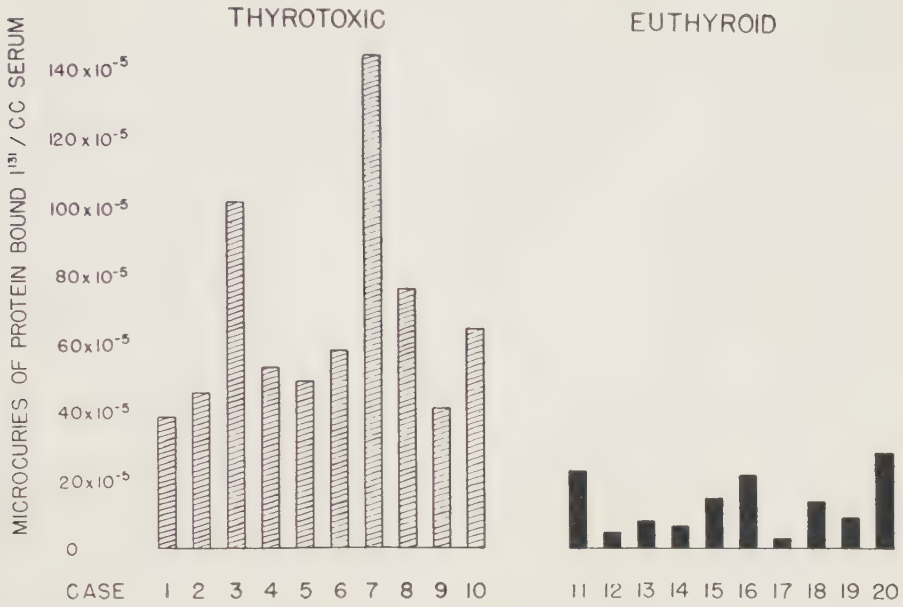

FIG. 1.
Serum Level Protein Bound I^{131}, 24 hours after standard oral dose of 150 μc I^{131}, carrier free, in 10 thyrotoxic and 10 euthyroid subjects.

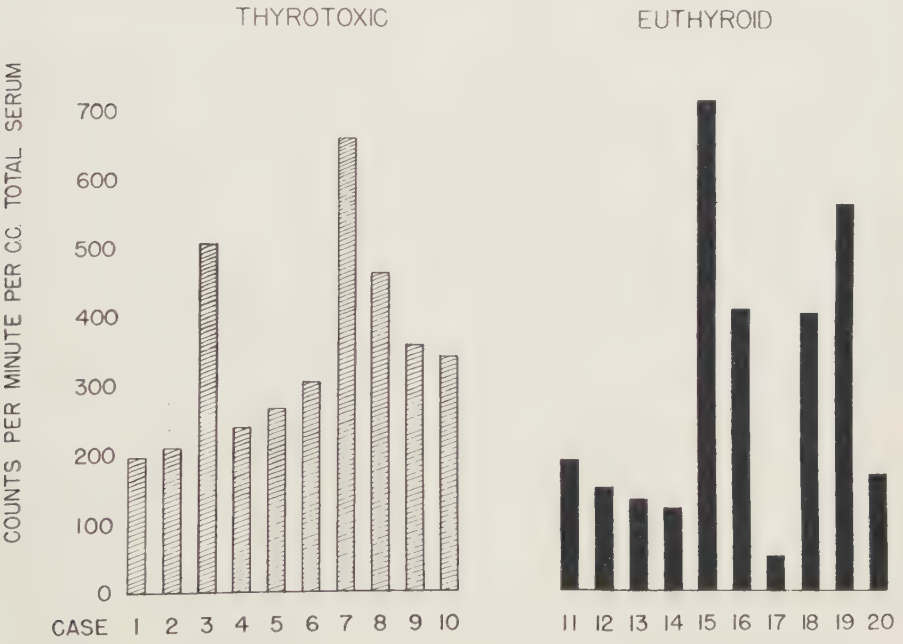

FIG. 2.
Counts/min./cc serum 24 hours after standard oral dose 150 μc I^{131}, carrier free, in 10 thyrotoxic and 10 euthyroid subjects.

after therapeutic doses (4-6 millicuries) showed that the protein bound I^{131} level at 24 hours was 50% higher than at 6 hours. The protein bound I^{131} serum levels at 48 and 72 hours, however, were increased only irregularly over the 24-hour concentration.

Summary. 1. The serum protein bound radioactive iodine (I^{131}) level was determined 24 hours after the oral administration of 150 microcuries I^{131}. Twenty subjects were studied; 10 were thyrotoxic and 10 euthyroid. The protein bound I^{131} was determined by a modification of the method described by Chaikoff *et al.*[8]

2. The serum protein bound I^{131} in thyrotoxic patients was 38 to 146 $\times$ 10^{-5} microcuries/cc averaging 68. In euthyroid subjects, the serum level ranged from 3 to 28 $\times$ 10^{-5} microcuries/cc, averaging 13.

3. It would appear that this test may be of diagnostic value as a measure of thyroid function.

17033

Inactivity of Bound Plasma Progesterone.*

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Observations have been reported indicating that progesterone in blood is in the plasma and not in the cells and that most of the total progesterone is free while rarely more than 10% is bound to protein or some other substance or substances that render this portion of the progesterone insoluble in acetone and ether.[1] The latter circumstance contrasts with the finding that approximately two-thirds of the estrogen in blood is bound.[2–4] It has been suggested that bound estrogen serves in effect as a reservoir, that the estrogen readily dissociates at the cell membrane, and that the bound fraction is potentially active estrogen.[3] Several observations indicate that a comparable situation does not obtain with respect to bound progesterone. The relative amount of the bound fraction seems too small to serve as a significant source of free progesterone; when introduced directly into the uterus of the mouse the bound fraction had no effect in a test period of 48 hours, presumably sufficient time to permit dissociation if it is to occur; the activity of raw plasma is identical with that of the free progesterone it contains. In short, bound progesterone appeared to be biologically inert.

When progesterone is introduced into the spleen or portal vein and must pass through the liver before reaching the systemic circulation the absorbed progesterone is inactivated in the sense that no effects of the substance are observed in rats,[5] rabbits,[6,7] and mice.[8] If the blood of such animals should contain levels of bound progesterone that would be effective if free, it would constitute further and probably strong evidence for the biological inactivity of the bound fraction. Such an experiment is described here.

Experimental. Young adult mice were used as test animals; the inbred A strain of Strong[9] was chosen to minimize variability. Of a group of 22 animals ovariectomized 13 days earlier, seven were given progesterone pellets subcutaneously, eight were given progesterone

* This study was aided by grants from the Committee for Research in Problems of Sex, National Research Council, and from the James Hudson Brown Memorial Fund of Yale University School of Medicine.

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pellets intrasplenically, and 7 were reserved as untreated controls. Of a group of 20 mice ovariectomized 47 to 49 days earlier, ten were given progesterone pellets subcutaneously, and 10 were given progesterone pellets intrasplenically.

The pellets were made of crystalline progesterone‡ and weighed 1 to 3 mg. Each animal received a single pellet. The subcutaneous pellets were implanted on the flank by means of a trocar and without anesthesia. The intrasplenic pellets were implanted under sodium amytal-ether anesthesia; splenic hemorrhage was controlled by packing wedges of muscle in the wound.

Four days after implantation of the pellets the mice ovariectomized at the beginning of the shorter period were bled by cardiac puncture. The blood from the members of each of the three subgroups (untreated, with subcutaneous pellets, with intrasplenic pellets) was pooled. Six, 8, 12, 16 and 22 days after implantation of the pellets two mice ovariectomized at the beginning of the longer period and carrying intrasplenic pellets, and 2 carrying subcutaneous pellets, were bled by cardiac puncture. The bloods from mice with the same histories were pooled. Sodium citrate was employed as the anticoagulant. Each pooled sample was centrifuged, and the free and bound fractions of progesterone in the plasma were separated by modification[1] of the method of Szego and Roberts[10] for separation of free and protein-bound estrogen. Briefly, the method involved precipitation of the plasma proteins with 10 volumes of acetone in the cold. The acetone solution contained the free progesterone, and the bound progesterone was freed by partial acid hydrolysis of the proteins. The two fractions were dissolved in sesame oil, and the progesterone content of each was assayed by intra-uterine injection in mice, a method that detects 0.0002 μg.[11]

After bleeding each mouse was autopsied, and the uterus was fixed in Lavdowsky's fluid[12] and prepared for microscopic study.

Findings. The pellets were intact and correctly located. No splenic adhesions were encountered in the animals carrying intrasplenic pellets. The uteri of most of the mice with subcutaneous pellets were of varying shades of yellow and slightly but distinctly enlarged; the uteri of the mice that had carried intrasplenic pellets for 22 days also had this appearance. The uteri of the other treated mice were like those of the untreated animals.

Microscopically, the endometria of the untreated mice showed no evidence of the action of progesterone; the stromal nuclei were shrunken, fusiform, and dense. Similarly, the mice carrying intrasplenic pellets showed no effects of progesterone upon the endometrium. The endometria of all of the mice carrying subcutaneous pellets for eight days or longer showed characteristic progestational changes in the stromal nuclei; they were enlarged, spherical, vesicular, and had distinct nucleoli.[13] When the subcutaneous pellets had been in place only four or six days no change in the stromal nuclei from the castrate condition was evident.

Assays of plasma from the untreated castrates revealed no free progesterone and at most a trace of bound progesterone.

The values for plasma progesterone in the mice carrying pellets are shown in Fig. 1. The animals carrying subcutaneous pellets had plasma levels that increased with the length of time the pellet was in place; indeed, the total progesterone levels (the sum of the free and bound fractions) fluctuated about a straight line when plotted against time. The level of free progesterone attained before an endometrial response was given was roughly 1.0 μg per ml.

The animals with intrasplenic pellets, on the other hand, had consistently low levels of free progesterone, the highest being 0.7 μg per ml. The bound progesterone, however, soon reached a high level in these animals, 2.0 μg per ml by the eighth day after implantation of

‡ The progesterone was generously supplied by Dr. Erwin Schwenk of the Schering Corporation.

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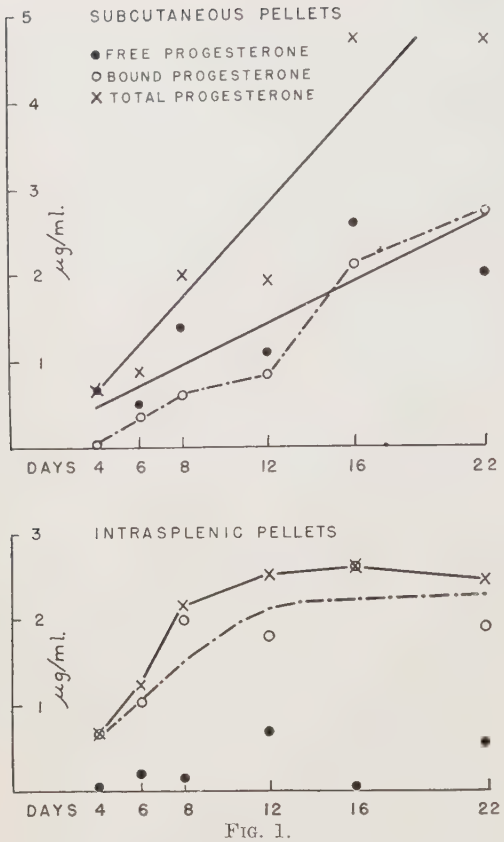

FIG. 1.

Plasma levels of free, bound, and total progesterone in ovariectomized mice with subcutaneous pellets and intrasplenic pellets of progesterone.

the pellets. Here the total plasma progesterone, in contrast to that of the animals with subcutaneous pellets, reached a plateau on the eighth day.

Discussion. All of the observations to date are in agreement in indicating biological inactivity of bound progesterone. The level of this fraction has been consistently low in various ovarian states;[1,14] amounts that would be effective if free have had no effect upon the endometrium when injected directly into the uterus of the mouse;[1] the effectiveness of raw plasma has been the same as that of the free progesterone it contains;[1] in the present experiment circulating levels of bound progesterone that regularly elicited endometrial responses when free were without effect even when present for presumably as long as 16 days.

The identity of the substance to which bound progesterone in the plasma is attached is unknown. It may be protein, as believed for estrogen.[2,3] On the other hand, the method employed for fractionation would probably not distinguish protein-bound material from conjugated material, such as a sulphate or a glucuronide, inasmuch as such conjugants would presumably be precipitated along with the plasma proteins by excess acetone. Whatever its nature, the binding appears to be firm and to dissociate slowly. This is suggested by the lack of response when bound progesterone is injected directly into the uterus and the endometrium is examined 48 hours later, presumably an adequate time for dissociation if it is to occur.[1] No or slow dissociation is further indicated by the present observation that levels of bound progesterone as high as 2.7 μg per ml may be present in the plasma for presumably as long as 16 days without dissociation of enough free progesterone to evoke a recognizable response in the endometrium. Accordingly, binding seems to serve no useful function in the circulation or availability of progesterone.

On the other hand, binding of progesterone is at least one of the methods of inactivating this hormone when it is absorbed in the spleen of the mouse. These observations do not show whether the binding and inactivation occurred in the liver or in the spleen, or indeed in the portal vein. Other studies,[5-7] however, have shown that the liver can inactivate this steroid, and have not revealed appreciable inactivation by the spleen. So far as we are aware, hepatic inactivation of steroids has been found in the past to involve alteration of the steroid compound.[15-20] Whether bind-

[14] Forbes, T. R., and Hooker, C. W., unpublished.

[15] Heller, C. G., *Endocrinology*, 1940, **26**, 619.

[16] Schiller, J., *Endocrinology*, 1945, **36**, 7.

[17] Pearlman, W. H., Paschkis, K. E., Rakoff, A. E., Cantarow, A., Walkling, A. A., and Hansen, L. E., *Endocrinology*, 1945, **36**, 284.

[18] Samuels, L. T., McCaulay, C., and Sellers, D. M., *J. Biol. Chem.*, 1947, **168**, 477.

[19] Levy, H., *Arch. Biochem.*, 1947, **14**, 325.

[20] DeMeio, R. H., Rakoff, A. E., Cantarow, A., and Paschkis, K. E., *Endocrinology*, 1948, **43**, 97.

ing of an apparently otherwise unaltered steroid compound is a method of inactivation peculiar to progesterone has not been determined.

No explanation can be offered at present for the apparent plateau in bound and total progesterone beginning eight days after implantation of the intrasplenic pellets. Obviously, the rate of absorption may have stabilized or declined, or the rate of removal of bound progesterone from the circulation by excretion or chemical alteration may have increased. Another possibility is that of increasing hepatic inactivation of the progesterone as absorbed by means other than binding.

The apparently linear relation of free and total plasma progesterone to time after the implantation of subcutaneous pellets also raises problems. One of the more interesting of these is the increase in the level of bound progesterone in these animals, possibly suggesting eventual hepatic inactivation of part of the absorbed material by binding. Although it may prove to be of no significance, it is interesting that the maximal level of bound progesterone was almost identical in the two groups of animals.

An interesting fact that emerges is that the minimal physiologically effective plasma level of progesterone with respect to the endometrium appears to be of the order of 1.0 μg per ml of the free fraction. Every animal with this or a higher level exhibited progestational changes in the stromal nuclei. All animals with lower plasma levels showed no endometrial response, irrespective of the level of bound progesterone.

Summary. Pellets of crystalline progesterone implanted into the spleens of ovariectomized mice had no effect upon the endometrium despite the presence of relatively high levels of bound progesterone in the plasma; the highest level of free progesterone in these animals was 0.7 μg per ml. Subcutaneous pellets of progesterone produced characteristic progestational changes in the endometrium when the level of free plasma progesterone exceeded 1.0 μg per ml. Binding appears to be a mechanism of hepatic inactivation of progesterone in the mouse.

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Effect of Dialyzed Enterogastrone Upon Twelve-Hour Nocturnal Gastric Secretion in Man.*

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Previous studies[1] have demonstrated occasional decreases in the 12-hour (nocturnal) and 24-hour gastric secretion in man following the intramuscular injection of 1000 to 3000 mg of an enterogastrone concentrate. This effect was variable in degree and temporary in duration. The administration of 400 to 2000 mg did not alter significantly the secretory response of the human stomach to the single standard dose of histamine or to the repeated injection of small amounts of histamine.[2] Insulin-stimulated secretion likewise was not affected, although the response possibly was modified by doses of 2000 or 3000 mg. Ferayorni, Code, and Morlock[3] similarly noted no change in gastric secretion during a double histamine test in 10 human subjects given 200 mg of enterogastrone intramuscularly. In 14 volunteers, quantities up to 400 mg intramuscularly and 18 g orally

* This study was supported in part by a grant from the Upjohn Co., Kalamazoo, Mich.

1 Kirsner, J. B., Levin, E., and Palmer, W. L., *Gastroenterology*, 1948, **10**, 256.

2 Levin, E., Kirsner, J. B., and Palmer, W. L., *Gastroenterology*, 1948, **10**, 274.

3 Ferayorni, R., Code, C. K., and Morlock, C. G., *Gastroenterology*, 1948, **11**, 730.

did not diminish the response to a modified Ewald test meal. Pollard and his associates[4] administered 8 to 16 g of the concentrate by mouth daily to 12 patients with peptic ulcer for as long as 11 months; 16 individuals received intramuscular injections of 200 mg daily for intervals up to 9 months. There were no changes in the volume of fasting gastric secretion, output of hydrochloric acid, concentration of pepsin or gastric motility.

The enterogastrone utilized in the initial experiments, although less crude than earlier products, consisted of a mixture of proteins of varying molecular size; its chemical composition is not known, and its physiologic characteristics are obscure. The most recent preparation of enterogastrone† is described[5] as free of inactive protein by dialysis through a cellophane membrane. Inhibition of gastric secretion in the rat and the dog approximated twice the degree obtained with the standard preparation. Neither this product nor other samples of enterogastrone have manifested anti-secretory activity in the guinea pig. Tests for toxicity are reported as satisfactory at 500 mg/kg and for vasodepressor activity as meeting present requirements.

The present study was undertaken to determine the effect of this concentrate upon the nocturnal gastric secretion of patients with peptic ulcer.

Method of study. The procedure was the same as that described in previous papers.[6-8] Ten male patients, 9 with active duodenal ulcer and one with a benign gastric ulcer, were studied. Constant suction of the gastric content was maintained by a Gomco aspirator. The volume and free acidity of each hourly collection were measured and the output of hydrochloric acid in milligrams calculated from these data. In 8 patients, the stomach was aspirated continuously for 60 hours, beginning at 9:30 P.M. After 48 hours had elapsed the enterogastrone was administered at 9:30 P.M. in single total doses of 200 to 5000 mg. The initial 12 hours (9:30 P.M. to 9:30 A.M.) constituted the control nocturnal period; the 12 hours (9:30 P.M. to 9:30 A.M.) after the injection of the concentrate represented the test period. In one case the control interval extended during the night and morning (11:30 P.M. to 11:30 A.M.); 2000 mg of enterogastrone then were given and the hourly secretion measured for the subsequent 12 hours (11:30 A.M. to 11:30 P.M.). A similar procedure was followed in the tenth patient; the control period was of 10 hours duration (11:30 P.M. to 9:30 A.M.); 5000 mg of enterogastrone then were administered and the gastric aspirations continued for the succeeding 10 hours (9:30 A.M. to 7:30 P.M.).

The patients were not given food or liquid by mouth. Normal electrolyte and fluid balances were maintained by the intravenous administration of 5% glucose in isotonic saline solution. The enterogastrone was injected intramuscularly into the glutei in a single dose. Pain developed locally almost at once, and usually was so intense as to prevent sleep. The body temperature remained normal in 7 cases; a slight elevation to 37.6 or 37.8°C occurred in 3 individuals (A.H., W.W., and C.M.) during the final one or two hours of the test period. No other untoward effects were observed.

Results. The data are recorded in Table I. The 12-hour gastric secretion was not reduced significantly in the four patients receiving 200 to 2000 mg of enterogastrone. The output of acid was decreased in each of the 6 individuals given 5000 mg of the preparation. The inhibition was slight in one and moderate or pronounced in 5. Depression of gastric secretion became apparent immediately in one case and within 2 or 3 hours in the remaining 5 patients. Anacidity for periods of 5 to 9

[4] (a) Pollard, H. M., Block, M., and Bachrach, W. H., *Proc. Central Society Clinical Research*, 1946, **19**, 34. (b) Pollard, H. M., Block, M., Bachrach, W. H., and Mason, J., *Arch. Surg.*, 1948, **56**, 372.

[5] Hailman, H. F., and Visscher, F., personal communication.

† Supplied by Dr. H. F. Hailman, Upjohn Co., Kalamazoo, Mich.

[6] Levin, E., Kirsner, J. B., Palmer, W. L., and Butler, C., *Arch. Surg.*, 1948, **56**, 345.

[7] Kirsner, J. B., Levin, E., and Palmer, W. L., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 108.

[8] Levin, E., Kirsner, J. B., and Palmer, W. L., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 153.

TABLE I.

Effect of Dialyzed Enterogastrone on 12-Hour Nocturnal Gastric Secretion of 10 Patients with Peptic Ulcer.

Case	Control			Amt enterogastrone intramuse. (mg)	After enterogastrone			Comment
	Vol. (cc)	Free HCl (Cl. units)	Total output HCl (mg)		Vol. (cc)	Free HCl (Cl. units)	Total output HCl (mg)	
♂—27 D.U.	976	79	2815	200	1114	77	3138	No effect
♂—42 D.U.	1033	49	1829	500	1047	38	1449	No significant effect
♂—39 D.U.	923	81	2719	1000	1130	85	3492	No effect
♂—51 D.U.	814	73	2487	2000	1054	70	3088	No effect
♂—57 D.U.	1056	87	3340	5000	1099	56	2646	Slight decr. 2 hr after inj.; reduced acid 2 hr., anacidity 2 hr
♂—34 D.U.	822	74	2218	5000	762	39	1071	Marked decr.; anacidity 3 hr after inj., continued for 7 hr
♂—72 D.U.	1647	48	2974	5000	1151	2	107	Marked decr. apparent immed.; anacidity 11 of 12 hr
♂—54 D.U.	1494	95	4874	5000	725	22	1251	Marked decr. after 3 hr.; anacidity 7 to 12 hr
♂—64 D.U.	1684	101	6220	5000	703	31	1501	Marked decr. after 3 hr.; anacidity 5 hr
♂—43 G.U.	519	12 (10 hr)	260	5000	332	6 (10 hr)	108	Moderate decr.; anacidity after 2 hr

consecutive hours was noted in 5 instances; in one case there was no free acid in 11 of the 12 specimens. However, hydrochloric acid usually reappeared towards the conclusion of the nocturnal period. Free acid had been present continuously during the control periods in the 9 patients with duodenal ulcer. The inhibition of secretion consisted usually of a pronounced decrease in the concentration of acid. However, the volume of gastric content also diminished markedly in 3 patients.

Comment. The present data indicate that the fasting gastric secretion may decrease greatly, albeit temporarily, following the intramuscular administration of 5000 mg of a dialyzed preparation of enterogastrone. This finding is in contrast to a single previous experiment in which gastric secretion was unaffected by the injection of 5000 mg of an apparently less concentrated product. Although the injections produced severe pain immediately, the decrease in acid usually did not become apparent until two or three hours had elapsed. As in preceding studies, the effect consisted chiefly of a lowered concentration of acid; the volume of secretion also diminished significantly in three of the present series. The mechanism of this reduction and its significance remain obscure. It may be noted that the intramuscular administration of a non-specific protein, sterile lactalbumin, did not reduce the nocturnal gastric secretion in 10 patients with peptic ulcer; indeed, an increased output of acid was noted in 6.[9] The inhibition is not attributable to an elevation in body temperature, since the temperature increased in only three patients and then very slightly; the reduction in acid in one of this group was slight. The temporary duration of the inhibition, the tremendous quantities required, the accompanying severe pain, and the uncertainty as to the nature of the material and the mechanisms involved do not warrant further clinical trial of the present dialyzed preparation of enterogastrone. Nevertheless, the data would appear to justify the continued search for a much more effective and safely administered product, whose chemical nature and physiological behavior may be studied more precisely.

Conclusions. 1. The output of hydrochloric acid in the 12-hour nocturnal gastric secretion of 4 patients with peptic ulcer was unchanged following the intramuscular administration of 200 to 2000 mg of a dialyzed preparation of enterogastrone. 2. Gastric secretion was reduced slightly in one and markedly in five of six patients with peptic ulcer, given 5000 mg. The transitory inhibition consisted chiefly of a decrease in the concentration of acid and, to a lesser extent, of the volume of secretion.

[9] Kirsner, J. B., Levin, E., and Palmer, W. L., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 90.

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Thiosemicarbazide: A New Toxic Derivative of Thiourea.*

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Previous papers from this laboratory[1-4] have described the acute toxicity of thiourea and a number of its singly substituted derivatives, notably alpha-naphthyl thiourea (ANTU), which has proved to be an effective rodenticide for the field control of Norway rats.[1] The subject of the present paper is thiosemicarbazide ($NH_2NHCSNH_2$), in

* Carried out under a contract between the Medical Division, Chemical Corps, U. S. Army, and The Johns Hopkins University. Under the terms of this contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the author.

TABLE I.
Acute Toxicity of Thiosemicarbazide.
(Adult animals, except as noted).

Species	No. used	Administered*	LD_{50} ± S.E.† mg/kg B.W.
Wild Norway rat *(Rattus norvegicus)*	Adult 56	s.t.	13 ± 2.1
	Young 21	"	19 ± 1.3
Laboratory rat *(Rattus norvegicus)*	Strain I 49	"	11 ± 2.0
	Strain II 44	"	18 ± 2.0
Alexandrine rat *(Rattus rattus)*	26	"	23 ± 1.4
Cotton rat *(Sigmidon hispidus hispidus)*	31	"	16 ± 2.2
Guinea pig *(Cavia cobaya)*	37	i.p.	24 ± 2.0
Dog *(Canis familiaris)*	6	s.t.	10 (5-15)
Cat *(Felix libyca domestica)*	4	"	20 (15-?)

* s.t. = by stomach tube; i.p. = by intraperitoneal injection. The vehicle was, in every instance, a 10% solution of gum acacia in water.

† LD_{50}'s and their standard errors were obtained by the method of Litchfield and Fertig.[7] Whenever data were not sufficiently extensive to justify statistical treatment, an estimate of the LD_{50} is followed by the range between the highest dose observed to kill none and the lowest dose killing all.

which an amino group replaces the naphthyl radical of ANTU. This compound differs in several respects from the other toxic thioureas, owing presumably to the fact that it is a derivative of hydrazine (NH_2NH_2) as well as of thiourea (NH_2CSNH_2).

The rapid death of white rats following administration of thiosemicarbazide was first observed by Dr. Emanuel Waletzky of the American Cyanamid Company; Dr. R. O. Roblin of that company then sent a sample to this laboratory for testing on wild Norway rats as a possible new rodenticide. Observations that this substance was toxic have also been made by Astwood[5] and by Jensen and Kjerulf-Jensen[6] in the course of their studies on the chronic feeding of various chemicals to determine their goiterogenic activity.

Acute Toxicity. Table I summarizes the data obtained with the 6 different species of animals available for assay. The thiosemicarbazide was administered either by stomach tube or by intraperitoneal injection, in water containing 10% gum acacia, according to the technic previously used for assaying ANTU.[3] Two strains of laboratory Norway rats were used to compare with the wild Norways, because previous work had shown that very marked differences in susceptibility to thiourea existed between domestic rats from different colonies.[2] The rats designated as strain I came from our own colony, which shows uniformly high susceptibility to thiourea poisoning; while those of strain II came from a colony of Wistar rats maintained at the Army Chemical Center, Edgewood, Maryland. The wild Norway rats were freshly trapped speci-

[1] Richter, C. P., *J. A. M. A.*, 1945, **129**, 927.

[2] Dieke, S. H., and Richter, C. P., *J. Pharm. and Exp. Therap.*, 1945, **83**, 195.

[3] Dieke, S. H., and Richter, C. P., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 22.

[4] Dieke, S. H., Allen, G. S., and Richter, C. P., *J. Pharm. and Exp. Therap.*, 1947, **90**, 260.

[5] Astwood, E. B., *J. Pharm. and Exp. Therap.*, 1943, **78**, 79.

[6] Jensen, K. A., and Kjerulf-Jensen, K., *Acta Pharmacol.*, 1945, **1**, 280.

[7] Litchfield, J. T., Jr., and Fertig, J. W., *Bull. Johns Hopk. Hosp.*, 1941, **69**, 276.

mens from the alleys of Baltimore. The Alexandrine and cotton rats came from our own colonies. All the rats were fed purina fox chow while in this laboratory.

Table I shows that all the acute median lethal doses obtained fell between 10 and 25 mg/kg. For comparison, the equivalent values for ANTU ranged from 2.5 mg/kg for domestic Norway rats up to about 500 mg/kg for cats (and over 4 grams/kg for monkeys and chickens).[3] This indicates that thiosemicarbazide does not share with ANTU a specific toxicity to Norway rats, but is more widely toxic to warm blooded animals in general.

One monkey (*Macaca mulatta*) was also available for testing the toxicity of thiosemicarbazide; it received 200 mg/kg by stomach tube and died within 24 hours.

The acute toxicity of thiosemicarbazide to young Norway rats was tested, since previous work showed that young rats were 6 or 7 times less susceptible to ANTU than were adults.[3] Thiosemicarbazide was given to 21 young wild Norway rats weighing between 36 g and 110 g and all sexually immature. The LD50 obtained with this limited number of rats (See Table I) was less than twice that found for adults, which indicates that compared to ANTU, the response to thiosemicarbazide is substantially less variable with age. This is borne out by the results of acceptance tests, described below.

Both male and female animals of every species were used at most assay levels. A separation of the data gave no indication of any difference in response that could be attributed to sex.

In all the animals included in Table I, with the significant exception of some laboratory Norway rats, the effects noted after poisoning were very similar. Within an hour after dosing the animals appeared to be in a highly excited and apprehensive condition. The rats and guinea pigs frequently squealed, the cats miaowed, and the dogs barked or growled. Violent convulsions then started, often terminating within a minute or two with the teeth bared and the limbs stiffly extended, only to begin again within a matter of minutes. Intense salivation was observed in all animals receiving fatal doses, and vomiting in the dogs, cats, and monkey. Death or what appeared to be complete recovery occurred within 6 hours, except for the monkey which survived for almost a day.

On the other hand, laboratory Norway rats of both strains often reacted differently to thiosemicarbazide, especially after low but nevertheless fatal doses. Those from our colony (Strain I) frequently had only mild convulsions or none at all, and deaths usually did not occur until after 18 to 24 hours. At autopsy these rats showed pulmonary edema accompanied by pleural effusion (as found in typical ANTU or thiourea poisoning). The other laboratory rats (Strain II) usually died in convulsions, but some of these also had none and died overnight with pulmonary edema.† In none of the other animals receiving thiosemicarbazide (including the monkey) was any pleural effusion found, nor was any significant amount of pulmonary edema evident either grossly or in histological sections of the lungs. This interesting finding is reminiscent of results previously reported,[2] in which poisoning of rats from our colony with the parent compound thiourea produced pulmonary edema, while equivalent doses had no effect on the lungs of wild Norway rats.

Acceptability. The voluntary consumption of thiosemicarbazide appears to be good, on the basis of tests made with individually caged rats. A concentration of 0.5% in either water solution or in a bait of yellow cornmeal killed 18 of 19 wild Norways, all of 8 Alexandrines, and 7 of 8 cotton rats. At 1% in bait all of 12 wild Norways died, and at 2% 7 out of 8. With 20 laboratory Norways (Strain I), divided into 3 groups, all of 8 died after drinking a concentration of 0.25% in water, and 4 of 8 at a 0.1% concentration, while none of 4 died from drinking a 0.05% solution. No hesitation to drink these solutions was observed, despite the fact that thiosemicarbazide has a bitter taste to humans and most probably also to rats.[8]

† Dr. Waletzky states (private communication) that rats from his colony, originating from Carworth Farms for the most part, exhibited violent convulsions and died in 1 to 3 hours. These rats would thus seem to resemble wild Norways more than the rats from our colony.

Of the 39 wild Norway rats included in this test, 29 were immature, weighing between 54 and 130 g. All but one of these young rats died after ingesting doses as low as 15 mg/kg, which indicates that this poison should be effective against young as well as adult wild Norway rats.

Tolerance. Thirty-six adult wild Norway rats and 6 adult laboratory Norway rats (Strain I) were used to investigate the possible development of tolerance to thiosemicarbazide. The wild Norways all received an initial dose of 7.5 mg/kg by stomach tube (approximately ½ an LD50 dose). Seven received 4 subsequent doses of 7.5, 7.5, 20, and 50 mg/kg, also by stomach tube, on alternate days thereafter. Convulsions were observed in 2 of these 7 rats following the 20 mg/kg dose, but all survived. All died within 3 hours after receiving 50 mg/kg, in violent convulsions.

Fourteen other wild Norways received 30 mg/kg on their second dose: 3 out of 3 died when the interval between doses was 14 days, 4 out of 4 when 3 days had elapsed, but only 3 out of 7 at 2 days after the initial poisoning.

When the second dosing was accomplished by offering poisoned water (0.25%) for voluntary consumption, 4 out of 4 died when the interval was one day, and at 2 days 7 out of 10. No wild Norway survived a dose higher than 60 mg/kg at any time. It would seem, then, that a tolerance to 4 or 5 median lethal doses can develop in wild Norway rats, and that this tolerance is most marked about 2 days after a sublethal dose. This is not serious from a practical standpoint, as the consumption of 5 cc of poisoned water (at 0.25%) or a few grams of poisoned food would provide ample thiosemicarbazide to offset such a tolerance, even in large sized rats.

In contrast to the above results, somewhat larger tolerances were established in the laboratory rats (Strain I). These rats received by stomach tube an initial dose of 5 mg/kg (or about ½ an LD50) and subsequent doses on alternate days of 5, 5, 20, 50, 100, 150, 200, and 300 mg/kg. One rat died (with pulmonary edema) from the initial dose, but no other fatalities occurred until 2 of the remaining 5 succumbed to 100 mg/kg. Two others died following 200 mg/kg and the last after 300 mg/kg. Thus 1 rat developed a tolerance to about 20 times the LD50 dose, on a regimen which with ANTU produces tolerances of 80 times with little difficulty, even in wild Norway rats.[1]

Antidote. The copious salivation found in animals poisoned with thiosemicarbazide suggested that atropine might be an antagonist. Preliminary experiments with 8 rats, one dog and one cat were unsuccessful; in fact, although the salivation was suppressed, the animals appeared to die rather sooner when treated with amounts of atropine which were not lethal to controls than when they received the thiosemicarbazide alone.

On the other hand the administration of 20 mg/kg of sodium pentobarbital at the onset of the first violent convulsion (about one hour after poisoning) followed by another similar amount when necessary, was effective in preventing the death of 2 dogs and one cat, each of which had received 5 times an LD50 dose. The convulsions were not entirely prevented by this amount of pentobarbital, but their intensity was lessened. Six hours after poisoning all 3 animals looked well although a little groggy; the next day recovery appeared to be complete.

The treatment of 16 laboratory rats (Strain II) with 40 mg/kg of sodium pentobarbital (and subsequent booster doses when necessary), at the onset of convulsions following either 3 or 6 LD_{50}'s of thiosemicarbazide produced some interesting results. Whereas the controls all died within 3 hours, none of the rats kept unconscious by the barbiturate died within the first 5 hours. Only 3 out of 8 survived the lower dose, however, and only one of the 8 receiving the higher dose: the rest died overnight with pulmonary edema and pleural effusion. It would therefore appear that pentobarbital is an antidote for those species in which thiosemicarbazide has only a convulsant action, but not when it also has an ANTU-like action on the lungs. Since 2 dogs survived relatively large doses when treated with this barbiturate, it may be that, unlike ANTU, thiosemicarbazide would not

[1] Richter, C. P., in press.

cause pulmonary edema in this species as it does in the laboratory Norway rat.

Comparison with other poisons. Some of the effects produced by thiosemicarbazide indicate the relationship of this compound to hydrazine. According to Fränkel,[9] hydrazine causes excitement and occasionally convulsions in higher animals, but does not damage erythrocytes as does phenyl hydrazine. O. Loew[10] reports convulsions and rapid death in guinea pigs after fatal doses of hydrazine. Underhill and Karelitz,[11] who studied the anemia caused in dogs by hydrazine sulfate, observed more or less constant salivation, vomiting and diarrhea; they mention no convulsions, however, nor does Bodansky[12] whose dog also vomited and salivated for several days.

To check the relative effects of thiosemicarbazide and the two compounds from which it derives, 14 laboratory rats (Strain II) were given thiourea and 14 hydrazine (as the sulfate), at the same time and in the same way that the LD50 of thiosemicarbazide was determined. The acute LD50 for thiourea fell at about 20 mg/kg, and that for hydrazine sulfate at about 450 mg/kg.‡ None of the rats receiving either thiourea or hydrazine sulfate had convulsions; the main autopsy findings in those succumbing to thiourea were pulmonary edema and pleural effusion, while hydrazine sulfate caused increased salivation and a grossly evident liver damage. A chocolate brown blood, as found after poisoning with phenyl hydrazine, was not observed.

The differences between thiosemicarbazide and the other toxic thioureas previously studied are several. Thiosemicarbazide has a strong convulsant action, which ANTU and other similar thioureas do not share, and appears to be quite generally toxic to a number of species of animals, rather than almost specifically toxic to Norway rats. It is not markedly less toxic to young than to adult Norway rats, and sublethal doses do not produce the high tolerances characteristic of ANTU. Furthermore, pentobarbital gives promise of being an adequate antidote, at least for those species not affected by the thiourea moiety of the molecule.

From a practical standpoint, thiosemicarbazide might have a wider applicability than ANTU because it dissolves in water and so can be used as a water poison as well as in bait. It can also presumably be used against other rodents than wild Norway rats: an evaluation of the success of such use must await field trials. On the other hand it is very probably toxic to man as well as to other animals and will therefore require far more caution in use. It acts very quickly and apart from a bitter taste, similar to that of phenyl thiourea and probably also subject to genetically controlled "taste blindness",[13] carries with it no warning. The existence of an antidote under these circumstances is comforting but may not be of much practical value.

Compared to other universal poisons such as sodium fluoroacetate (1080), the value of thiosemicarbazide would seem to lie in its lower toxicity, which under some circumstances might be an advantage, since it would lessen the hazard to larger animals, including man. The consumption of a few grams of bait is sufficient to kill rats, and since the animals feel unwell within a short time after poisoning they rarely take more than that. No individual rat in our tests has voluntarily consumed sufficient poison to kill a (much larger) dog or cat by secondary poisoning, assuming no loss of toxicity of the substance while in the rat's body.

Thiosemicarbazide is a relatively stable compound under ordinary conditions of light-

[9] Fränkel, S., *Die Arzneimittel-Synthese*, 6th ed., Berlin, 1927, pp. 84-85.

[10] Loew, O., *Ber. d. Deut. Chem. Ges.*, 1890, **23**, 3203.

[11] Underhill, F. P., and Karelitz, S., Jr., *J. Biol. Chem.*, 1923, **58**, 147.

[12] Bodansky, M., *J. Pharm. and Exp. Therap.*, 1924, **23**, 127.

‡ This last finding confirms a previous report[4] as to the relatively low toxicity of hydrazine sulfate to laboratory rats, and also agrees well with results obtained with a few wild Norway rats, for which strain the LD_{50} probably falls between 250 and 500 mg/kg.

[13] Blakeslee, A. F., and Fox, A. L., *J. Hered.*, 1932, **23**, 97.

ing, humidity and temperature. It melts at 180°C with decomposition, but below that temperature it appears not to decompose spontaneously. It is soluble in cold water to about 1 or 2% and in hot water to about 10%. It is also soluble in alcohol.§

Summary. Thiosemicarbazide, a derivative of both thiourea and hydrazine, has proved to differ from other toxic thioureas previously studied. Instead of producing fatal pulmonary edema in a limited number of animal species and not harming others at equivalent dose levels, thiosemicarbazide caused convulsions and death within 1 to 3 hours in the 6 species tested, when given in amounts ranging from 10 to 30 mg/kg. In some individual laboratory rats, however, including those in which the convulsions had been suppressed by administration of a barbiturate, death was delayed and accompanied by the development of pulmonary edema. Thiosemicarbazide may have promise as a practical rodenticide, because of its general toxicity to rats of several species and because it appears to be readily accepted in lethal amounts when offered to rats in either water solution or bait.

§ The analytical data on thiosemicarbazide given in this paragraph were very kindly provided by Dr. J. R. Vaughan of the American Cyanamid Company.

It is a pleasure to acknowledge the kindness of Dr. Wayland G. Hayes of the Communicable Disease Center, U. S. Public Health Service, Savannah, Georgia, who provided the nucleus of our Alexandrine rat colony, and of Dr. R. O. Roblin, American Cyanamid Company, who has helped in many ways throughout the course of this study.

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Estimation of Dicumarol, 3, 3¹-Methylenebis (4-Hydroxycoumarin) in Biological Fluids.*

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Although the physiological activity of dicumarol has been assayed by its effect on the prothrombin time, no chemical method for its estimation has been available. A rapid chemical method would make possible a study of certain problems concerning dicumarol about which little is known. These are its physiological disposition in man, its relationship to vitamin K in the clotting mechanism, and its formation in spoiled sweet clover.

A chemical method is described below which involves isolation of the drug from the body by extraction into heptane. The drug is returned to alkali and measured spectrophotometrically at 315 mμ where the drug exhibits a pronounced peak (Fig. 1).

Reagents. 1. Standard solution of dicumarol 100 mg per liter. 100 mg of dicumarol are dissolved in 1 liter of 0.1 N NaOH. This solution is stable for at least one month when stored in the refrigerator.

2. 3 N HCl.

3. Heptane. (Paragon Testing Company). A technical grade of heptane is purified by successive washings with 1 N NaOH and 1 N HCl followed by 2 washings with water.

4. 2.5 N NaOH.

Procedure. Add 1 to 3 ml of plasma or urine (sample containing up to 50 γ of dicumarol) and 0.5 ml of 3 N HCl to 20 ml of heptane in a 60 ml glass-stoppered bottle. Adjust the aqueous volume to about 3.5 ml if necessary by the addition of water. Shake for 30 minutes on a shaking apparatus and

* This work was supported by a grant from the Institute for the Study of Analgesic and Sedative Drugs.

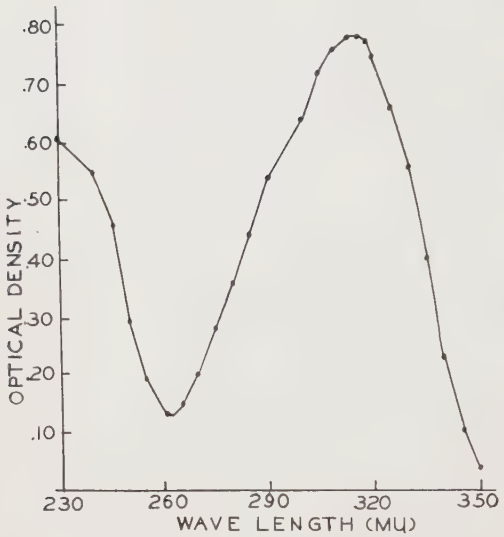

FIG. 1.

The absorption spectrum of dicumarol in 2.5 N NaOH. The concentration of the compound was 10 γ per ml. Cell thickness = 1 cm.

then centrifuge the bottle. Transfer 15 ml of the heptane phase to a 60 ml glass-stoppered bottle containing 4 ml of 2.5 N NaOH. Shake for 5 minutes. Transfer the contents to a test tube and centrifuge for 5 minutes. Remove the organic phase by aspiration with a fine-tipped pipette. Transfer about 3 ml of the aqueous phase to a quartz cuvette and determine the optical density in a spectrophotometer (Beckman) with the instrument set at the wave-length 315 mμ. A reagent blank with water substituted for plasma is run through the same procedure. This blank is used for the zero setting and should have an optical density not greater than 0.005 when 2.5 N NaOH is used for the zero setting.

Standards. The distribution of dicumarol in a heptane-acidified water system is such that at room temperature with volumes of 20 and 3.5 ml respectively, about 95% of dicumarol is in the organic phase. Standards are prepared by handling known amounts of dicumarol as in the procedure described above. The optical densities were found to be proportional to concentration. An optical density of about 0.130 is obtained in a Beckman spectrophotometer when 10 γ of dicumarol are run through the above procedure.

Results. Recoveries of dicumarol added to plasma in amounts of 5 to 50 γ were quite satisfactory (100 $\pm$ 3%). The sensitivity is more than adequate for the plasma levels which are obtained after therapeutic doses of the drug.

Assay of Specificity. There is a negligible amount of material in normal plasma and urine which assays as dicumarol in the analytical procedure described above. The possible interference by metabolic products of the drug was examined by a distribution technic previously described by one of us.[1,2] It involves a comparison of the distribution of the substance extracted from plasma with that of the authentic substance in a two-phase system consisting of an organic solvent and water at various pH values. Dissimilar distributions indicate the presence of a substance different from the authentic compound. To escape detection a transformation product would have to have not only a similar dissociation constant but identical solubility characteristics in two solvents.

The examination in the case of dicumarol was made with heptane extracts of the pooled plasma of 2 subjects who had received the drug. The plasma was obtained 23 hours after the oral administration of a 500 mg

TABLE I.

Distribution of Dicumarol and Apparent Dicumarol Between Heptane and Water at Various pH Values.

The apparent dicumarol was obtained by extraction with heptane of the acidified plasma of 2 subjects who had received dicumarol. The compound was returned to dilute alkali. Aliquots of this solution and of an authentic dicumarol solution were adjusted to various pH values and shaken with 2 volumes of heptane. The fraction of the compound extracted at various pH values is expressed as the ratio of the amount of compound in the organic phase to total compound.

pH	Authentic dicumarol	Apparent dicumarol from plasma
1	.95	.94
6	.93	.91
6.5	.90	.87
7.0	.81	.80
7.5	.53	.52
8.0	.36	.35

[1] Brodie, B. B., and Udenfriend, S., *J. Biol. Chem.*, 1945, **158**, 705.

[2] Brodie, B. B., Udenfriend, S., and Baer, J. E., *J. Biol. Chem.*, 1947, **168**, 299.

dose. The distributions of dicumarol between heptane and water at various pH values were compared with those of the apparent compound extracted from plasma. The results showed that within experimental error the apparent and authentic compound had the same solubility characteristics and were, therefore, presumably the same compound (Table I).

Basic organic drugs do not interfere in the procedure for dicumarol since they are not extracted at an acid pH. The following acidic or neutral drugs were tested for their interference in the procedure for dicumarol: acetanilide, phenacetin, antipyrine, phenobarbital, sulfanilamide, sulfadiazine, sulfathiazole, penicillin, vitamin K, nembutal, pentothal, and salicylic acid. Only pentothal and salicylates interfered in the procedure and consequently these substances should not be present when analyses for dicumarol are being made.

Summary. A simple and sensitive spectrophotometric method for the estimation of dicumarol in plasma and urine is described. Dicumarol is isolated from the biological material by extraction into heptane. The drug is returned to alkali and measured spectrophotometrically at 315 mμ. The method is specific in that it does not include metabolic products of the drug.

17037 P

Seasonal Variations in the Choline Content of Human Serum.

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In the literature there are but few reports of studies on serum choline content (Guggenheim and Loffler,[1] Sieburg and Patzschke,[2] Luecke and Pearson,[3] and Schlegel[4]).

It appears from these studies that the concentration of choline in human serum ranges from about 0.2 to 2 mg %. As far as has been ascertained, no records are available of studies presenting evidence of seasonal variations in serum choline content.

The present study comprises 142 duplicate determinations of serums obtained from both men and women in the period from May 1, 1947 to May 1, 1948. Determinations of choline were made according to the method of Abdon and Ljungdahl-Ostberg[5] by converting choline into acetylcholine, which physiologically is about 100,000 times as active as choline. Its action was determined

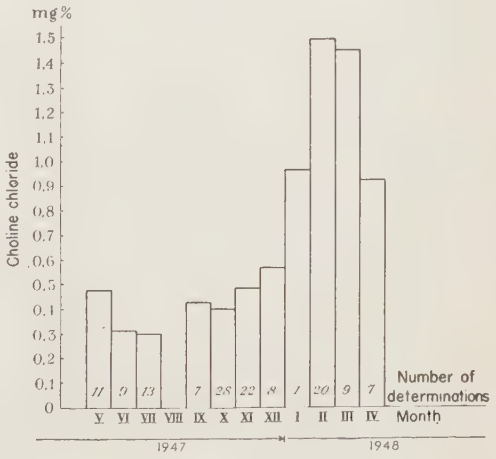

FIG. 1.

The average serum choline content during the different months of the year.

1 Guggenheim, M., and Loffler, W., *Biochem. Z.*, 1916, **74**, 303.

2 Sieburg and Patzschke, *Z. d. ges. exp. Med.*, 1923, **36**, 324.

3 Luecke, R. W., and Pearson, P. B., *J. Biol. Chem.*, 1944, **153**, 259.

4 Schlegel, J. U., Variationer i Serumcholinindholdet hos Mennesker, Copenhagen 1948 (Thesis).

5 Abdon, N. O., and Ljungdahl-Ostberg, K., *Acta Physiol. Scandinav.*, 1944, **8**, 103.

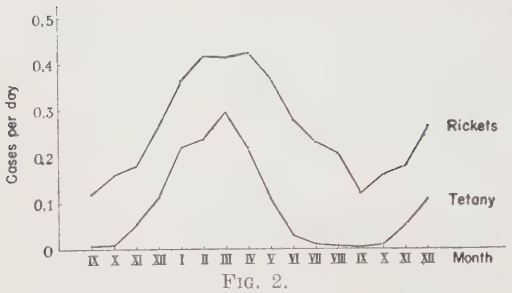

FIG. 2.

Monthly distribution of the incidence of rickets and tetany during the years 1924-35 incl. The material is from Borne-hospitalet, Fuglebakken, Copenhagen.[6]

biologically on intestines of guinea pigs. The accuracy of the method is 12% on single determinations, 9.4% on duplicate determinations.[4]

Fig. 1 shows the results of these determinations for choline over a 12-month period.

It will be seen that the average choline content is lowest in the month of July (0.3 mg %), the concentration being 5 times as high in the months of February and March.

The cause of these seasonal variations is not known, but we wish to call attention to the remarkable correspondence of the serum choline curves in Fig. 2, showing seasonal variations in the incidence of rachitis and tetany (Horstmann and Petersen[6]).

With the view of ascertaining the relationship between the choline seasonal curve and the curves (Fig. 2) of minimum accumulation of light,* which is secondarily manifested by deficiency of vitamin D, a few experiments were carried out in which the response of the serum choline level to irradiation was studied. Table I shows the results obtained in these experiments.

It appears from the 5 irradiation experiments that the serum choline content dropped quite considerably subsequent to about three weeks of carbon-arc light irradiation . It should be pointed out, however, that the last 3 experiments were carried out in the latter part of April when the average choline content had already dropped.

From the present studies it seems justifiable to reckon with the possibility that the variations in serum choline content within the various months of the year may be related to the effect of light accumulation in the same way as is the case with vitamin D, except, however, for showing an opposite course, with minimum values in the winter months of February and March, instead of the summer months.

It is impossible, on the basis of the present

TABLE I.

Results Showing the Effect of Carbon Arc Light Irradiation on the Serum Choline in Five Different Persons.

	Case	Course of light treatment			Final effect
Date of initiation of irradiation and subsequent choline determinations	a	3/2/48	3/2/48	3/10/48	3/22/48
	b		3/3	3/10	4/12
	c		4/2	4/ 8	4/22
	d		4/2	4/ 8	4/22
	e		4/2	4/ 8	4/22
Minutes of exposure to carbon arc light at two day intervals	a	0	4	14	30
	b		0	4	16
	c		0	10	24
	d		0	10	20
	e		0	6	20
Serum choline (mgm %)	a	1.37	1.51	2.14	.48
	b		—	1.41	.61
	c		—	1.33	.49
	d		—	2.55	.78
	e		—	1.94	.98

[6] Horstmann and Petersen, H., *Acta paed.*, 1947, **33**, 203.

* For full clarification of the term "light accumulation" the reader is referred to the work of Horstmann and Petersen.[6] In brief, it refers to a delayed, cumulative effect of light upon the organism.

studies, to evaluate the consequences of the observations made, in the same way as it seems difficult to understand the cause of the probable significance of light in relation to the concentration of choline in serum. It seems, however, that determinations for choline in serum might present a possibility of measuring—by a fairly simple method—the effect of light on the organism. Furthermore, the variations seem to be so large that a chemical method of determination might well be employed.

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The Pathogenicity of Bagasse, II. Effect on Rabbits of Prolonged Exposure to Bagasse.*

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The clinical and industrial health problems of Bagasse Disease have attracted increasing attention.[1–12] In spite of careful observation and biopsies on patients suffering of this disease, its pathogenesis remains obscure.[2]

Various factors have been suggested as etiologic agents: the particular composition of the fiber itself, fungi and micro-organisms attached to the fiber, and the high silica content. Allergic phenomena also entertained as a possible etiologic mechanism[3] seem, on the basis of experimental evidence,[4] less likely to be involved.

The problem of establishing whether the fiber itself, or the micro-organisms growing in abundance on it,[4,5] is responsible for the disease was approached experimentally by comparing the lesions produced by either untreated, autoclaved, or formalized bagasse.[4] In short term experiments a striking difference was observed. Rabbits developed a rapidly progressing, even fatal, disease after intravenous or intratracheal administration of fresh bagasse, while the autoclaved or formalized material produced only a foreign body reaction limited to the lungs. In the present report these experiments were extended in order to study the character of the lesions at longer intervals, to resolve the complex histopathology of the lesions into components that could be correlated with the various ingredients of bagasse dust, and to compare the morphology of the experimental lesions with that of the human disease.

Material and methods. For intratracheal insufflation, a procedure described earlier was employed.[4] The attempt to expose animals

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1 Anonymous, *J. A. M. A.*, 1948, 1050.

2 Lemone, D. V., Scott, W. G., Moore, S., and Koven, A. L., *Radiol.*, 1947, **49**, 556.

3 Castleden, L. I. M., and Hamilton-Paterson, J. L., *Brit. M. J.*, 1942, **2**, 478.

4 Gerstl, B., Tager, M., and Marinaro, N. A., *Arch. Path.*, 1947, **44**, 343.

5 Hunter, D., and Perry, K. M. A., *Brit. J. Industr. Med.*, 1946, **3**, 64.

6 Sonkin, L., Lipton, W., and Van Hoesen, D., *J. Industr. Hyg. and Tox.*, 1946, **28**, 273.

7 Gardner, L. U., *Am. Rev. Tuberc.*, 1920, **4**, 734.

8 Moore, M., *Arch. Path.*, 1946, **42**, 113.

9 Browne, C. A., *J. Am. Chem. Soc.*, 1904, **26**, 1221.

10 Hirsch, E. F., and Russel, H. B., *Arch. Path.*, 1945, **39**, 281.

11 Sodeman, W. A., and Pullen, R. L., *Arch. Int. Med.*, 1944, **73**, 365.

12 Koven, A. L., *Am. Rev. Tuberc.*, 1948, **58**, 55.

TABLE I.
Rabbits Exposed to Bagasse in Dusting Chamber.

Rabbit No.	No. of hours exposed	Duration of exposure, days	Day after last exposure that animal died (d) or was sacrificed (s)
296	270	108	11th (s)
297	270	108	21st (s)
347	30	6	1st (d)
356	72	15	1st (d)
359	90	15	1st (d)
362	180	44	24th (s)
352	30	6	30th (s)
363	180	44	36th (s)
348	54	12	36th (s)

to a continuous and somewhat controlled flow of dust for days or weeks met with difficulty. An arrangement, similar to that recommended by Sonkin *et al.*[6] proved unsuccessful because of the hygroscopic bagasse powder rapidly becoming sticky and plugging the jet. Shaking a large amount of bagasse and maintaining the dust in the air by means of a fan or blower resulted in unequal distribution in the dusting chamber. Finally, resort was taken to a modification of the relatively simple method employed by Gardner.[7] Bagasse was finely shredded in a Waring blender and strained through a wire gauze sieve, 150 meshes per inch. Dusting was carried out for various periods during the day time. (See Table I). Approximately 15 g of bagasse were expended during 6 hours of dusting. The animals with their fur thickly coated with dust were then replaced in their cages.

To secure ash, bagasse was kept in an electric furnace at 850°F, with air blown into the oven to insure oxygenation. The resulting ash was ground in a glass homogenizer. The average yield was 5.74%. Hunter and Perry[5] as well as Koven,[12] reported 3 to 4%. The silica content of the ash was 43.34 and 43.05%.‡ The ash of 10 g bagasse, ground with the addition of saline, was made up to 90 cc. Each cubic millimeter contained approximately 35,000 particles. Its total content of solid particles, estimated volumetrically after centrifugation, was twice that of suspension of bagasse.[4] The mineral content, however, was about 10 times that of the suspension of bagasse fiber.

An extract of the resinous substances was obtained by refluxing 5 g of shredded bagasse with 100 cc of petrol ether or benzol for 10 hours. The solvent was evaporated from the filtrate. The residue suspended well in 15 to 20 cc of water, to which 2 drops of monoethanolamine had been added.

Results. A. Intratracheal insufflation of bagasse. Rabbits studied at 20 and 35 days after the intratracheal insufflation of 10 ml of suspension of fresh bagasse showed frequent and large pneumonic lesions, with the alveolar exudate predominantly monocytic in character, though polymorphonuclears were numerous in places. The interstitium was infiltrated by small and large round cells. Fibroblastic proliferation was noted at the periphery of several lesions. Other lesions were of a foreign body granulomatous type and composed of multinucleated giant cells and mononuclears grouped together by bundles of fibroblasts. Cytoplasmic defects of irregular shape were often seen in the giant and mononuclear cells, but birefringent bodies could be identified under polarized light only in a few instances. From the pulmonary lesions of one of the rabbits (35th day) an Aspergillus was isolated on culture. In some sections radiate bodies (Moore[8]) could also be identified. Rabbits treated similarly with autoclaved or formalized bagasse and studied at identical intervals revealed lesions composed of one to several multinucleated giant and mononuclear cells, both showing cytoplasmic vacuoles. Slight fibroblastic pro-

‡ These values were obtained on a single sample and carried out by Dr. Carl Tiedcke, New York City.

liferation and a few small round cells were seen at the periphery of some of the lesions, rendering them granuloma-like. There was little difference between the morphology of these lesions and those observed at 10-day intervals.[4]

B. Effect of ash and resins of bagasse. For the purpose of differentiating the effect of minerals present in bagasse from that of the fiber as a whole, 2 rabbits received each 10 ml of ash suspension, as described under Materials and Methods, by the intratracheal route, and were sacrificed 35 days later. Microscopic sections revealed occasional foreign body giant cells, both single or in small groups, in the interstitial pulmonary tissue. Some irregular shaped cytoplasmic defects in these cells corresponded, under polarized light, to double refractile bodies. In the pulmonary sections of one of the animals two small granulomatous lesions, composed of a few multinucleated giant cells and fibroblastic proliferation were noted; and in a section of the other animal a few alveoli plugged by young connective tissue were noted. (Fig. 1).

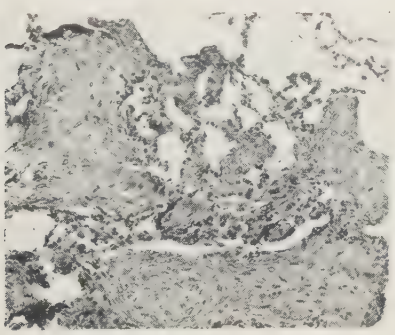

FIG. 1.
Rabbit 328. Treated intratracheally with ash of bagasse; 35th day. Lung. Masson strain. ×75.

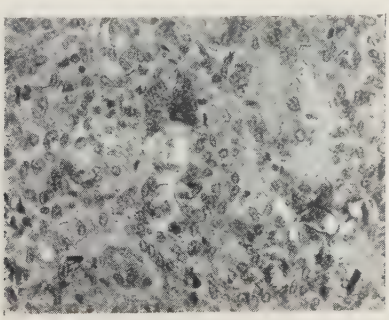

FIG. 2.
Guinea pig. Intraperitoneal injection of ash of bagasse; 90th day. Peritoneal tissue. H. & E. stain. ×165.

Intravenous administration of similar material in repeated small doses, totaling 8 ml produced occlusion of numerous capillaries by multinucleated giant cells which had formed around irregular shaped, sometimes highly refractile, foreign bodies. Similar giant cells were also found occluding lymphatics. This type of lesion was observed at 3 and 4 day intervals as well as at 20 and 35 days. In the latter group they differed only by being less numerous and by the occasional presence of a few small round cells or slight fibroblastic proliferation around the giant cells. The pulmonary changes in two rabbits, treated similarly and sacrificed at 55 days, were almost identical to those of the aforementioned ones. It is noteworthy that the other organs of these animals were free of lesions.

Three guinea pigs each received intraperitoneal injections of 2 cc of the ash suspension, and were sacrificed 90 days later. Two of them revealed granulomatous lesions attached to the visceral and parietal peritoneum, and composed of numerous large giant cells, mononuclears, and slight fibrous tissue proliferation (Fig. 2). Numerous variously shaped foreign bodies were within cytoplasmic vacuoles of these cells. None of these lesions resembled those seen in experimental silicosis.

The extent and number of lesions, as well as the intensity of the cellular reaction in the rabbits that received ash suspension were strikingly less than in the animals treated with autoclaved or formalized bagasse, although the mineral content of the material administered was a multiple of that in the former group.

Browne,[9] in an early investigation of the composition of bagasse, pointed to its high resin content. Resins are not innocuous substances, although the literature on this subject is scanty (Hirsch and Russel[10]). An attempt was, therefore, made to arrive at an appraisal of the biologic properties of the resins present in bagasse. Extracts, prepared as stated under Methods, and suspended in ethanolamine water were administered intra-

tracheally so that each of three rabbits received the total from 2.5 g of bagasse. The animals were sacrificed on the 35th day. A moderate number of multinucleated giant cells of the foreign body type were seen in the interstitial tissue of the lungs. Some of them contained large, irregularly shaped, foreign bodies which did not rotate the beam of polarized light. There was no other cellular reaction.

C. Exposure to bagasse dust. Animals were exposed to dust for periods indicated in Table I. For dusting rabbits 296 and 297, various mechanisms including the jet devised by Sonkin *et al.*[6] were employed. Only for the last 10 hours of exposure were these animals kept in the dusting chamber used for all other animals listed in Table I. Between the two animals there was a striking difference in type and extent of lesions. A few alveoli containing a mononuclear exudate were the only changes seen in rabbit 297. Although rabbit 296 was sacrificed 10 days later, its necropsy revealed extensive acute inflammatory changes. In the lung, numerous alveoli were filled by a polymorphonuclear and monocytic exudate with some of their nuclei showing pyknosis and karyorrhexis. Disintegrated cells were also noted in the perivascular lymphatic tissue. Foci of necrosis were present in liver and spleen.

Three animals, kept in the dusting chamber as indicated (Table I), died after 30, 72 and 90 hours of exposure. (Rabbits 347, 356 and 359). It is noteworthy that their exposure mates survived for a prolonged period. The microscopic preparations of the first rabbit revealed a hemorrhagic pneumonia; those of rabbit 359 revealed fairly numerous mononuclear cells in alveoli and in distended lymphatics. Small foreign bodies were seen in many exudate cells. Two healing myocardial infarcts may have accounted for the early death of this animal. The sections of rabbit 356 revealed an extensive pneumonic process with the exudate being polymorphonuclear in places, in others predominantly mononuclear. Many bronchioli were plugged by a similar, sometimes disintegrated, exudate. Occasional organization of the bronchiolar and bronchial exudate was found (Fig. 3). Multinucleated giant cells were infrequent. In addition to numerous small variously shaped foreign bodies there was noted an occasional club-shaped body, continuous with a fragment of a mycelial-like structure (Fig. 4). Frequently groups of large mononuclears with a foamy cytoplasm were seen both in alveoli and interstitium. Similar changes were also responsible for some polyp-like protrusions of the interstitium into the alveolar lumina. Some of the alveoli were lined by a cuboidal metaplastic epithelium.

Four animals were exposed to bagasse dust from 30 to 180 hours, and sacrificed at intervals from 24 to 36 days after the last exposure. These animals, because of the approximating interval, as well as the similarity

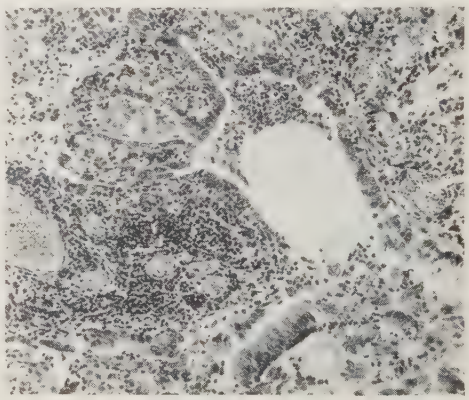

FIG. 3.
Rabbit 346. Exposed to bagasse dust for 72 hours. Died. Lung. H. & E. stain. ×100. Bronchioli plugged by exudate. Interstitial infiltration by inflammatory cells.

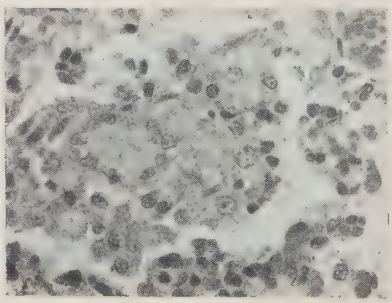

FIG. 4.
Rabbit 356. Lung. Masson stain. ×425. Spore and mycelia-like structure; foreign body giant cell.

of lesions, may be considered together. There were many alveoli in groups of 3-20 which were filled by closely packed large round or polygonal cells, some of which could be identified as mononuclear phagocytes. Their cytoplasm contained brown dust-like material and also birefringent rods. A few multinucleated giant cells of the foreign body type and occasional polymorphonuclears were present. There was no necrosis. In pulmonary sections of rabbit 363 there was a marked thickening of the interstitium by monocytic infiltration and fibroblastic proliferation sometimes forming protrusions (Fig. 5) similar to those observed in rabbit 356.

Three guinea pigs (337, 338 and 339) were also exposed in the dusting chamber for a total of 77 hours over a period of 18 days, and sacrificed 10 days later. The short interval was selected in order to observe any acute inflammatory changes which might have subsided at a later date. In the pulmonary sections some alveoli and bronchioli contained an exudate of large vacuolated mononuclear cells and an occasional polymorphonuclear. Elsewhere, large multinucleated giant cells were seen. Numerous foreign bodies were present within cells of both types. When compared with the lesions of rabbits exposed for a similar length of time, the almost complete absence of an acute inflammatory response in the guinea pig was striking.

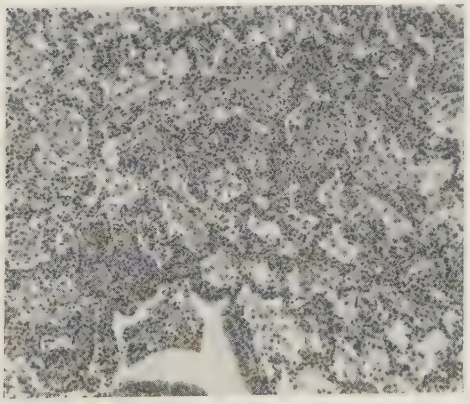

FIG. 5.

Rabbit 363. Dusted 180 hours, 36th day. Lung. H. & E. stain. ×60. Marked interstitial infiltration.

Discussion. The striking difference between lesions resulting from the administration of live bagasse, and those from bagasse dust with all living matter killed by either autoclaving or formalizing, as described first in short time experiments, was also manifest in animals kept alive for longer intervals. This is suggestive evidence that some living matter attached to the bagasse fiber, in itself an injurious agent, enhances the effect, and produces an additional pathologic change. The bagasse fiber, without living matter, elicits a long-standing foreign body reaction. Bagasse dust in the native state, when inhaled or insufflated, calls forth, in addition to the foreign body granuloma, an acute inflammatory response which, in view of its presence many days after the exposure, allows no other interpretation than that self-propagating microorganisms act upon the tissue of the host. For the rabbit, an aspergillus seems to be the pathogenic agent. It could be recovered from the pulmonary lesions 35 days after intratracheal administration of bagasse dust. This finding would support the theses of other authors[3,5,12] that fungi play an important part in the pathogenesis of the human disease.

Two components of the bagasse fiber could be demonstrated as being responsible for a long persistent tissue reaction, although the latter is of minor degree. One of them, the mineral particles, is of interest for the high silica content. But none of the lesions produced by the mineral ash resembled those of human or experimental silicosis. This would bear out the assumption of several authors that human Bagassosis is not caused by the high silica content of the bagasse fiber. The resins similarly contribute to the persistence of the lesions, as evidenced by the presence of giant cells 35 days after intratracheal insufflation. It also could be established that the resins, at least in their extracted form, do not contribute to the birefringence of the bagasse particles.

The most interesting part of the investigation was the effect of bagasse dust when inhaled by animals under conditions simulating those under which the human disease is acquired. It was soon apparent that some of the animals reacted violently to the dust with

instant and protracted cough, and developed a rapidly progressing pneumonia. The complex morphology of the lesions found in these animals includes hemorrhagic pneumonia, bronchiolitis, with plugging of passageways by cellular exudate and debris, interstitial thickening by exudate and fibroblastic proliferation, as well as of foreign body reaction. On the other side are the animals that offered a higher resistance, possibly aided by elimination of some particles by the upper respiratory channels. These animals probably would have survived indefinitely and demonstrated the ability to resolve gradually the lesions which they undoubtedly harbored at an earlier date.

Although these two groups of animals represent an interesting parallel to the selective morbidity among workers in the bagasse industry, a comparison of the morphology of the human with that of the experimental disease is hindered by the scarcity of human material available.§

Of the 2 cases that came to autopsy (Sodeman,[11] Hunter and Perry[5]) only one has been reported so far. Large spicules, as illustrated in Sodeman's case, could not be found in the tissue of animals that were exposed to the dust. Apparently particles of that size could not pass the smaller respiratory lumina of the animals. Other characteristic features are interstitial fibrosis, bronchiolitis,[5] and the presence of numerous large foamy alveolar cells filling the alveolar spaces.[11]

In the experimental lesions interstitial fibrosis was scanty. This may be due to a greater resolving power of the animal tissue or to the fact that the more susceptible animals succumbed too early (6 and 15 days) to have developed extensive fibrosis. Fibroblastic proliferation, however, was observed. Large foamy exudate cells and plugging of the bronchi were the features whereby the experimental most closely resembled the spontaneous disease.

The character of the lesions in the guinea pig differed strikingly from that in the rabbit. These represented a response to foreign bodies and lacked the acute inflammatory and progressive component, and are apparently due to the fact that the guinea pig is not susceptible to the organisms associated with bagasse.[4,5]

Conclusions. These experimental and comparative studies permit the conclusion that the inorganic part of the inhaled bagasse dust produces a long-standing tissue reaction which is primarily a foreign body response. It is unlike silicosis and amenable to healing by resolution. Superimposed on these lesions there occurs in those animals which are more susceptible to the causative microorganisms acute bronchiolitic and pneumonic changes which, if sufficiently extensive, may cause death of the animal. A similar combination of etiologic factors seems to be the most plausible explanation of the complex picture of human bagasse disease.

Summary. Bagasse irrespective of the route of administration, produces a complex and frequently progressive inflammatory reaction of the exposed animal. By employing native, in contrast to autoclaved bagasse and its extracted resins and minerals, it could be demonstrated that the fiber itself calls forth a long-standing foreign body reaction which is amenable to healing. The progressive inflammatory reaction and death of the animal, however, are due to microorganisms attached to the bagasse. In the instance of the rabbit, aspergilli are the pathogenic agents. The points of similarity of the experimental lesions to those observed in humans are discussed. None of the lesions resembled silicosis.

§ Dr. W. A. Sodeman kindly made available several slides of the pulmonary lesions of the case observed at Tulane University.

I wish to express sincere thanks to the Hospital Photographic Laboratory, Letterman General Hospital, San Francisco, for preparing these microphotographs.

17039

Complete Regression of Lymphosarcoma Implants Following Temporary Induction of Riboflavin Deficiency in Mice.

Herbert C. Stoerk and Gladys A. Emerson.

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It has been found previously that lymphosarcoma implants show marked regression following the administration of a pyridoxine antagonist (desoxypyridoxine).[1] In recent experiments a number of other vitamin antagonists were examined for their possible effect upon lymphosarcoma (6C3H-CD) in mice of the C3H strain. It was found that the feeding of a diet low in riboflavin produced regression of established lymphosarcoma implants. This regression was enhanced by the administration of riboflavin antagonists (isoriboflavin,[2] galactoflavin.[3]).

C3H male mice 5-6 weeks of age were inoculated with lymphosarcoma (6C3H-ED)* fragments under sterile precautions. The tumor transplants were placed under the skin of the lower back. The tumors were then permitted to grow for 6 to 14 days. During this time the animals were maintained on a complete diet of natural foods. They were then divided into experimental groups and subjected to treatment as indicated in the table. At the end of the feeding periods the animals were transferred back to the stock diet. The findings summarized in the table show that in 51 control animals on a stock diet (Group I), the tumors attained an average size of 9.2 cm^3 within 20 days. After 30 days there were no survivors in this group. Mice fed for 14 days (group II) and for 26 days (group III) a diet deficient in vitamin B_2 showed marked to complete regression of the established implants within 10 days. Thirty per cent of the mice in group II and 37% in group III survived for more than 60 days, a period after which no recurrences were ever observed up to 200 days. Amounts of riboflavin required for maintenance in C3H mice, when fed to lymphosarcoma bearing animals (Group IV), were not sufficient to permit growth of the implants at the usual rate. However the rate of tumor growth did not significantly differ from that in the controls (Group I) when 8 μg (Group IX) or 10 μg (Group V) were given daily to the animals. Tumors that were permitted to grow to a larger size (Group VIII) regressed rapidly when a riboflavin antagonist was administered. The feeding of a riboflavin analogue Group VI and VII) was rendered ineffective when 100 μg of vitamin B_2 were given simultaneously. Animals surviving for more than 60 days, when reinoculated with lymphosarcoma tissue, failed to "take" the second implant. The administration of other vitamin antagonists (pyrithiamine, 3-acetyl pyridine and pteroyl aspartic acid) had no significant effect upon the lymphosarcoma implants.

Regression of lymphosarcoma transplants, produced by the administration of a pyridoxine antagonist (desoxypyridoxine),[1] occurred more rapidly than the regression observed in riboflavin deficiency. However refeeding of vitamin B_6 deficient animals with pyridoxine was followed by recurrence of the tumors. In mice in which tumor regression had been induced by transitory vitamin B_2 deficiency, the tumor did not recur when riboflavin was fed. Moreover in these mice even reinoculation was ineffective. This suggests strongly that immunization may be an important contributing factor in the permanent suppression of the lymphosarcoma transplants. In the riboflavin deficient rat antibody formation is unimpaired whereas immune responses in pyri-

1 Stoerk, H. C., *J. Biol. Chem.*, 1947, **171**, 437.

2 Emerson, G. A., and Tishler, M., Proc. Soc. Exp. Biol. and Med., 1944, **55**, 184.

3 Emerson, G. A., Wurtz, E., and Johnson, O. H., *J. Biol. Chem.*, 1945, **160**, 165.

* The tumor was obtained through the courtesy of Dr. W. U. Gardner, Yale University, New Haven, Conn.

TABLE I.
Growth of Lymphosarcoma Implants and of Survival in the Various Experimental Groups.

Group	No. of mice	No. of days on defic. diet	μg fed		Tumor size in cm³ and survival in % at									
			B_2	Anti-B_2	10 days		20 days		30 days		40 days		50 days		60 days	
					cm³	%	cm³	%	cm³	%	cm³	%	cm³	%	cm³	%
I	51	—	60*	0	1.4	100	9.2	86	—	0	—	0		0		0
II	8	14	0	0	1.5	100	<1	100	0	37	0	37	0	37	0	37
III	10	26	0	0	0.8	100	0	100	0	90	0	70	0	60	0	30
IV	10	38	5	0	1.0	100	4.9	100	7.1	70	0	40	0	20	0	20
V	10	28	10	0	0.9	100	12.6	100	14.8	70	—	0	—	0	—	—
VI	10	14	3	500‡	2.5	100	<1	100	0	60	0	50	0	40	0	40
VII	10	14	100	500	1.3	100	11.5	90	—	0	—	0		0		0
VIII	10	20	8	1000§	—†	100	14.0	90	<1	70	0	30	0	30	0	30
IX	10	20	8	0	—†	100	15.3	90	15.8	10	—	0		0	—	0

* Complete natural diet.
† No measurement made.
‡ Isoriboflavin.
§ Galactoflavin.

doxine deficiency are suppressed.[4] It is probable that the recurrence of the tumor transplants following recovery from vitamin B_6 deficiency is due to the absence of immune bodies directed against the neoplastic tissue or its causal agent.

While pyridoxine deficiency produces marked atrophy of both normal[5] and neoplastic lymphoid tissue,[1] riboflavin deficiency affects only neoplastic lymphoid tissue. Riboflavin deficiency in the rat,[5] chick and mouse,[6] does not alter normal lymphoid tissue to a greater extent than does a comparable degree of inanition. The apparent exceedingly high requirement of neoplastic lymphoid tissue for vitamin B_2 may therefore be either a characteristic of the tumor lymphocyte or may perhaps represent a property of its causal agent, possibly a virus. The demonstration by Morris[7] that the growth of a mammary adenocarcinoma, related to a filtrable factor, is retarded by riboflavin deficiency, appears compatible with the latter possibility.

Summary. Marked regression of established lymphosarcoma (6 C3H-ED) implants occurred in all of 48 C3H mice rendered temporarily deficient in riboflavin either by the feeding of a diet low in this vitamin or by the administration of an antagonist. In most cases survival was significantly prolonged and 15 mice survived without recurrence of the tumors for more than 200 days. When animals which had survived 60 days or more were reinoculated with lymphosarcoma tissue, the second implant failed to take. Established lymphosarcoma implants in 81 control mice on a diet supplemented with adequate amounts of riboflavin, grew continuously and killed all animals within about 4 weeks.

[4] Stoerk, H. C., Eisen, H. N., and John, H. M., *J. Exp. Med.*, 1947, **85**, 365.

[5] Stoerk, H. C., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 90.

[6] Unpublished observations.

[7] Morris, H. P., *Ann. N. Y. Acad. of Sciences*, 1947, **49**, 119.

17040

Colloidal Properties of Nucleus. I. Effect of Temperature on Nuclear Viscosity in the Starfish Egg.

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Although the colloidal properties of the nucleus are important to an understanding of its role in cell division, very little information of a quantitative nature has been obtained from the living cell. It is evident from the observations of Gray[1] and Harris[2] that the nuclear contents of certain marine eggs are in a liquid state. Gray noted that the nucleolus in the germinal vesicle of the Echinus egg moved under the influence of gravity, and he determined the rate of its movement. Heilbrunn[3] was able to estimate the absolute viscosity by substituting the value for the rate of movement into Stokes' formula and assuming the densities of nucleoplasm and nucleolus on theoretical grounds. Harris[2] from his photographic observations on nucleolar movement calculated the absolute viscosity of the germinal vesicle of the Echinus egg to be about 10 centipoises, and he checked this value with determinations of viscosity by Pekarek's Browian movement method. He also found that a fall of the nucleolus was clearly seen in the eggs of 4 other species of echinoderms. It seems, therefore, that in the germinal vesicles of certain marine eggs, the viscosity is approximately 10 times that of water.

The purpose of this investigation is to determine the effect of temperature on nuclear viscosity in the egg of the starfish, *Asterias vulgaris*, by means of the falling nucleolus method. This method has the distinct advantage of yielding quantitative results without harming the egg in any way.

Procedure. Eggs in the immature germinal vesicle stage were used. The ovaries from freshly dissected animals were placed in sea water in fingerbowls, and the eggs, which were shed almost immediately, were kept at the temperature of running sea water (about 19°C) until used. Eggs removed from the animal for more than 3 hours were discarded. For each experiment a drop of dilute egg suspension was placed on a depression slide with a rectangular depression approximately 125 μ deep. A few strands of absorbent cotton were put in the chamber to keep the eggs in position and a coverslip lightly ringed with vaseline was placed on top. The eggs were then observed through a horizontal microscope.

After the suspension had remained a few minutes in this position, an egg was selected for observation. The microscope stage was then revolved 180° and the movement of the nucleolus through the entire diameter of the nucleus timed. In each observation the time was measured from the instant the stage was revolved until the nucleolus was observed to reach the bottom of the nucleus. This was repeated several times with each egg.

Temperature was varied by placing the microscope in a cold room to obtain temperatures between 3°C and 24°C. For higher temperatures the microscope was placed in a constant temperature box equipped with heating coils. Readings were made with the thermometer bulb directly on the stage. For each experiment the microscope was allowed to come to equilibrium with the desired temperature for 15 minutes or more.

Results. The results obtained are summarized in Table I, which shows the average number of seconds required for the nucleolus to fall at each temperature studied. When the values in the third column of Table I are plotted against temperature a straight line relation is suggested. The data were analyzed by the method of least squares. The resulting equation is:

1 Gray, J., *Brit. J. Exp. Biol.*, 1927, **5**, 102.

2 Harris, J. E., *J. Exp. Biol.*, 1939, **16**, 258.

3 Heilbrunn, L. V., *The Colloid Chemistry of Protoplasm*, Berlin, 1928.

TABLE I.
Effect of Temperature on Time of Fall of Nucleolus and the Thixotropy of the Nuclear Colloid.

Temp. °C	Time for initial fall, sec.	Avg of times for subsequent falls, sec.	Ratio initial time to avg. of subsequent times	No. of experiments	Total No. observations
3.5	285	230	1.239	1	8
8.9	235	204	1.152	1	7
10.5	273	223	1.225	3	30
12.5	220	199	1.103	3	12
14	215	158	1.360	1	10
16	200	167	1.188	6	39
17	185	153	1.294	3	22
19	165	137	1.205	3	14
23	150	142	1.056	1	5
24	144	130	1.105	7	49
25	133	122	1.097	9	58
27	122	128	0.957	8	52
28	104	112	0.941	5	51
30	95	102	0.983	1	12
33-35.5	120	81*		1	10
33-37	95	95†		1	2

* Nuclear colloid gelled after tenth reading.
† Nuclear colloid gelled after second reading.

$$Y = 245.1 - 4.865\ T$$

where Y = Time for nucleolus to fall through entire diameter of nucleus (seconds).
T = Temperature (degrees C).

The line defined by this equation is shown in Fig. 1, the plotted points representing averages of all determinations for each 5 degree interval. Since viscosity varies directly with the time, corresponding values for viscosity were included on the ordinate scale. These values were calculated from Stokes' formula by substituting 7.17×10^{-4} cm for nucleolar radius, 56.6×10^{-4} cm for the distance the nucleolus falls, and 0.1 for the difference in density between nucleoplasm and nucleolus. The latter value for difference in density is theoretical. Justification for this is given by Heilbrunn[3] in his monograph.

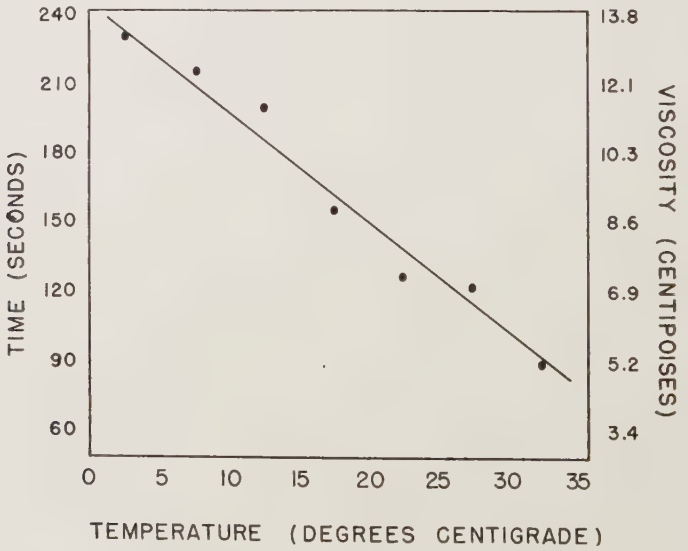

FIG. 1.
The effect of temperature on the time of fall of the nucleolus. Corresponding values for viscosity are on the right side of the graph.

TABLE II.
Results of Application of the t-test in Determining Significance of Deviations of Mean Differences from the Theoretical Value of Zero for Two Temperature Intervals.

	Temp. range (°C) 3.5-24.5	25.0-30.0
Mean value of differences for given temperature interval	28.75	—1.4
Deviation of mean value of differences from *O*	28.75	—1.4
t value	6.268	0.489
No. of experiments	28	20
Probability of obtaining such a deviation by chance alone from the theoretical mean of *O*	0.00	0.63

Ladenburg's correction factor was used. This corrects for the effect of the nuclear membrane on the velocity of the moving nucleolus. Although the equation has not been adapted for a sphere moving within a sphere, Harris from observations on a model nucleus deduced an empirical value of 0.29 for the Echinus egg nucleus. Since the relative proportions of nucleolus and nucleus are approximately the same in the Asterias egg as they are in the Echinus egg the same value was used in these calculations. As can be seen from Fig. 1, the absolute viscosity, determined in this way, is about the same as that found in the Echinus egg by Harris. It would appear, therefore, that at least in the temperature range investigated the absolute viscosity changes by approximately 2.7 centipoises with a 10°C change in temperature. This change is evident up to temperatures of approximately 35°C. In this range the nuclear contents gel, as is evident from the third column in Table I, which indicates that the nucleolus did not move at or above this temperature.

Also, from Table I, the thixotropic nature of the nuclear colloid is suggested. In the fourth column the mean ratio of initial reading to average of subsequent readings is given for each temperature employed. If the nucleoplasm is a thixotropic colloid, this ratio should be greater than one. This is found to be the case at lower temperatures. However, the values appear to decrease with increase in temperature until a ratio close to unity is reached. This is at a temperature of approximately 25°C. Apparently, therefore, temperatures of 25°C or above abolish thixotropy.

In order to test the significance of this apparent change in thixotropy the differences between the initial readings and the averages of subsequent readings were analyzed by means of the t-test (based on Student's distribution). The mean of the differences obtained in experiments at temperatures of 25°C through 30°C proves to be insignificantly different from zero. The mean of the differences corresponding to temperatures of 3.5°C to 24.5°C, however, is significantly greater than zero. The numerical results of these calculations are given in Table II.

Discussion. There were no marked deviations from a linear relationship between viscosity and temperature within the range studied. This is unlike the cytoplasmic reaction in the Cumingia egg as found by Heilbrunn.[4] In this case the viscosity goes through a maximum value at 15°C. In some other cells, however, the cytoplasmic viscosity decreases progressively as the temperature is raised until the protoplasm gels (see Heilbrunn[3] for discussion). This is similar to the results obtained with the starfish egg nucleus. Coagulation of the starfish egg nucleoplasm occurred at approximately 35°C. For the present data, the latter value is necessarily an approximation since a complete description of heat coagulation would involve not only the temperature but also the time that the eggs were exposed. Heilbrunn[5] has shown that the protoplasm of the Arbacia egg coagulates at 31°C to 37°C, that of the Cumingia egg at higher temperatures. In both cases, the temperature coefficient of the change in rate of coagulation per degree change in

[4] Heilbrunn, L. V., *Am. J. Physiol.*, 1924, **68**, 645.

[5] Heilbrunn, L. V., *Am. J. Physiol.*, 1924, **69**, 190.

temperature lies between 1 and 2. Investigations are planned to determine other properties of the nuclear colloid, particularly with regard to its radiosensitivity. The present knowledge of temperature effects will serve as a guide to the proper experimental conditions.

Summary. By means of the falling nucleolus method it was determined that a 10°C increase in temperature lowers nuclear viscosity in the Asterias egg by approximately 2.7 centipoises. Thixotropic properties disappear at temperatures above 25°C and coagulation occurs at about 35°C.

17041

Effect of Stretch and Pressure on Stimulus Formation in the Dog's Auricle.

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The application of a few crystals of aconitine or of 0.05 cc of a 0.05% solution of aconitine to the auricular appendix of the exposed heart of the dog results in a regular tachycardia with a rate of 300-500 beats per minute. Invariably this tachycardia responds to faradic stimulation of the vagus nerves with an increase of rate.[1] The administration of aconitine to the site of the sinus node also leads to a tachycardia which spontaneously or following stimulation of the vagus or sympathetic nerves is transformed into auricular fibrillation.[2,3] Because of its high rate and the ease of its transformation into fibrillation the auricular tachycardia was considerd to be auricular flutter. Cooling the small area to which the aconitine has been applied stops the fibrillation (or flutter); it reappears immediately when cooling is interrupted.[2] Earlier it was pointed out that these observations are not compatible with the assumption that auricular fibrillation is due to a circus movement mechanism. An extremely rapid stimulus formation with secondary formation of reentry waves was considered the responsible mechanism.[2]

This report deals with observations made during application of stretch and pressure on the right auricle of the dog's heart in the presence of an aconitine-induced tachycardia.

Method. The technic employed was the same as in previous investigations. The heart was exposed with the dog under nembutal anesthesia and artificial respiration. Aconitine was injected into the region of the head of the sinus node. All electrocardiograms were recorded in lead II. Stretching of the auricle was accomplished by one of several methods: 1) by attaching a weighted hook to the right auricular appendix or the right auricle at the junction between the vena cava superior and the right appendix; 2) by stretching the tip of the appendix of the right auricle by means of a blunt forceps; 3) by rapid intravenous infusion of 50 cc of 0.9% saline at body temperature into the jugular vein or the vena cava superior. Pressure on the area into which aconitine had been injected was exerted by a probe. In all experiments we satisfied ourselves that the site of application of aconitine actually was the focus of stimulus formation because cooling of this site invariably resulted in a cessation of flutter or fibrillation as long as cooling continued.

Results. In 11 experiments stretching led to an increase of auricular rate and to auricular fibrillation. The fibrillation sometimes developed so quickly that the transitional period of tachycardia could not be analyzed. More often a short period of increased rate preceded the fibrillation. The duration of the

* Istanbul, Turkey.

1 Scherf, D., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 233.

2 Scherf, D., Romano, F. J., and Terranova, R., *Am. Heart J.*, 1948, **36**, 241.

3 Scherf, D., *Am. Heart J.*, in press.

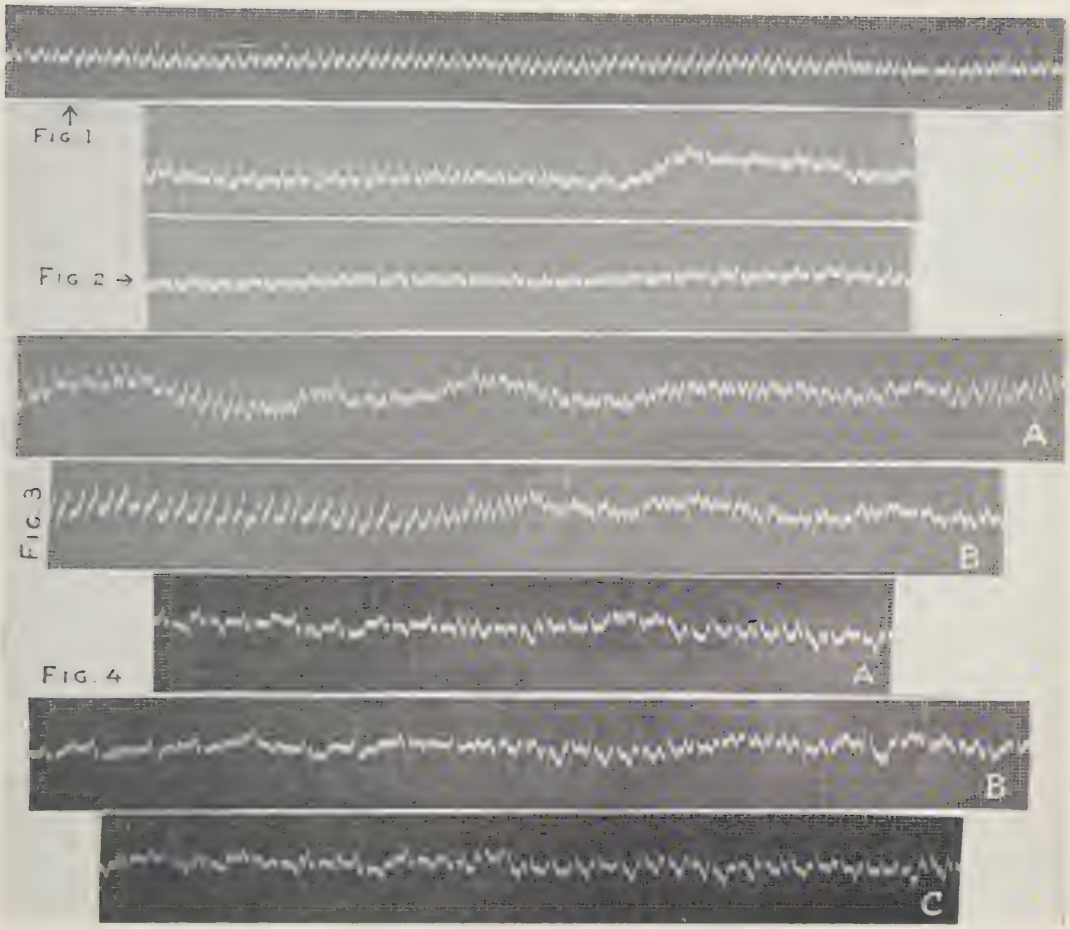

FIG. 1. Stretching of the right auricle changes auricular flutter into fibrillation.

FIG. 2a and b. The tracings are continuous. Stretching of the right auricle causes temporary auricular fibrillation and return to flutter.

FIG. 3a and b. Stretching of the right auricle causes a marked increase of the flutter rate and fibrillation.

FIG. 4a, b and c. In Fig. 4a stretching changes sinus rhythm into transient auricular fibrillation; rapid dilatation of the right auricle caused by intravenous injection has the same result (Fig. 4b). Pressure exerted on the site of injection causes auricular fibrillation (Fig. 4c).

fibrillation thus elicited varied from a few seconds to minutes.

Fig. 1 was obtained in an experiment on Sept. 23, 1947. Application of the aconitine solution to the head of the sinus node caused an auricular flutter with a rate of 460 per minute and a 2:1 A-V block. Stretching of the auricle with a weight of 20 g increased the auricular rate to 540 and finally to 600; fibrillation then suddenly appeared.

In the experiment of May 6, 1948 auricular flutter with a rate of 400 and an irregular A-V block was elicited in a similar manner. With stretching the rate increased to 500 and then to about 600; at this moment fibrillation immediately followed (Fig. 2a). Fig. 2b represents the direct continuation of Fig. 2a and shows the return of auricular flutter after approximately 10 seconds.

In the experiment of Jan. 25, 1949 the fibrillation induced by stretching lasted only about 7 seconds (Fig. 3a). The auricular rate before the stretching at the beginning of Fig. 3a measures 420 per minute. This rate was increased with stretching to about 600 and again fibrillation appeared. With the

return of flutter, at the end of the tracing, the auricular rate is again 600 and gradually fell to values of around 460. The stretching was repeated about 12 minutes later with the same result. The auricular rate increased from 420 to about 660 beats per minute and auricular fibrillation appeared (Fig. 3b).

Of particular interest are the tracings of Fig. 4. In this experiment auricular stretching had converted auricular flutter into fibrillation which persisted for 58 minutes. The stretching was performed shortly after the injection of aconitine and it is possible that the stretching hastened the appearance of fibrillation which would have developed anyway. After the disappearance of fibrillation repeated stretching of the auricle by traction on the appendix regularly elicited transient fibrillation.

Fig. 4a shows a regular sinus rhythm with a rate of 126. Stretching caused auricular fibrillation after the auricular rate had increased from 200 to 300. Two P waves which are situated within the QRS complex just before fibrillation started, follow each other with a rate of about 600. Shortly after this fibrillation subsided, 50 cc of saline were rapidly injected into the jugular vein in order to cause acute dilatation of the right auricle. Fig. 4b obtained during this maneuver shows that after a short period of sinus node inhibition and A-V escape beats fibrillation developed. Repetition of this experiment twice yielded the same results. Finally pressure was exerted on the site of the injection of aconitine with a blunt probe and fibrillation reappeared immediately (Fig. 4c).

Similar observations were made in other experiments for a period of 15-20 minutes after the disappearance of flutter or fibrillation.

Discussion. In all 11 experiments identical results were obtained. If the auricular muscle containing the sinus node into which aconitine had been injected was stretched by traction or by rapid intravenous introduction of fluid invariably an increase of auricular rate and then auricular fibrillation were observed. Even after the aconitine induced fibrillation (or flutter) had disappeared they could be reinduced by these measures.

The appearance of rhythmic stimulus formation or the increase of rate of an already existing stimulus formation in a response to the mechanical stimulus of stretch or pressure is known to exist in sensory nerves, in motor nerves, the skeletal muscle, the heart muscle of the frog[4] and the bundles of specific fibers of the heart of the dog.[5] In lower animals, the mollusks for example, the stretch caused by cardiac filling is the physiologic stimulus for cardiac activity. Stretching and pressure facilitate depolarization of the cell membrane;[6] stronger mechanical stimuli of this kind cause local injury of the type seen during continuous chemical or electrical stimuli which also lead to rhythmic stimulus formation. On the other hand conductivity does not seem to be altered by mechanical stretching. This fact has been demonstrated for the skeletal muscle,[7] for the junctional A-V fibers of the heart of the frog[8] and finally for muscle strips from the frog's ventricle.[9] In the experiments mentioned last stretching which increased by 20% the distance between two points on which the electrodes were attached did not increase conduction time.

In view of the fact that the mechanical devices used in our experiments influence only the formation of stimuli and not the conduction of the impulses we see in these observations further justification for the assumption that in auricular flutter and fibrillation we are dealing primarily with a disturbance of stimulus formation. The rate of stimulus formation is increased by stretching and when a certain limit is reached—in our experiments a rate of about 600 per minute—fibrillation appears. There are no data available which permit us to assume

[4] Gaskell, W. H., *J. Physiol.*, 1880, **3**, 48.

[5] Goldenberg, M., and Rothberger, C. J., *Arch. f. d. ges. Physiol.*, 1935, **235**, 597.

[6] Adrian, E. D., and Gelfan, S., *J. Physiol.*, 1933, **78**, 271.

[7] Schenck, F., *Arch. f. d. ges. Physiol.*, 1896, **64**, 179.

[8] Engelmann, T. W., *Arch. f. d. ges. Physiol.*, 1894, **56**, 149.

[9] Schellong, F., *Z. f. Biol.*, 1925, **82**, 451.

that stretching causes an increased rate of conduction of a circus wave. As soon as the rate of stimulus formation reaches values of approximately 600 per minute the appearance of refractory islands of muscle makes a regular response impossible and flutter changes into fibrillation.

The appearance of fibrillation following stretching, particularly on stretching by intravenous infusion (Fig. 4b) is of interest because it may explain the attacks of paroxysmal fibrillation on sudden physical exertion. The increased filling of the right auricle particularly at the beginning of sudden severe physical exercise[10] may in a predisposed heart elicit paroxysmal fibrillation.[11-13]

We fully appreciate the fact that the assumption of a rapid stimulus formation as the cause of auricular flutter or fibrillation[2] will necessitate an answer to one pertinent objection: Why does stimulation of the vagus nerves increase the auricular rate of one type of auricular tachycardia (auricular flutter) and stop the stimulus formation during sinus rhythm or other auricular tachycardias? Auricular extrasystoles caused by the intravenous injection of minute doses of aconitine disappear during the faradic stimulation of the vagus nerves. They increase in number after the stimulation of the vagus is discontinued.[14] At the present time we are not prepared to offer an explanation for this fundamental difference. It is possible that local application of aconitine causes the establishment of a continuous stimulus; this would elicit more frequent responses with a shortening of the refractory phase during stimulation of the vagus nerve. If extrasystoles occur due to discontinuous stimulus formation vagal stimulation inhibits them.

Summary. Stretching of the auricular muscle fibers during a tachycardia induced by aconitine leads to an increase of rate and to transient auricular fibrillation. Transient auricular fibrillation (or flutter) can be elicited by stretching during a period of 15-20 minutes after the aconitine arrhythmia has subsided and sinus rhythm prevails.

Because there is much evidence that stretching changes impulse formation and not impulse conduction these results are believed to bring further evidence against the circus movement hypothesis.

The experiments offer an explanation for the appearance of paroxysmal fibrillation induced by physical exertion.

[10] Eyster, J. A. E., The clinical aspects of venous pressure, Macmillan, New York, 1929.

[11] Hay, J., and Jones, H. W., *Brit. M. J.*, 1927, **1**, 559.

[12] Orgain, E. S., Wolff, L., and White, P. D., *Arch. Int. Med.*, 1936, **57**, 493.

[13] Jervell, O., *Acta med. Scandinav., Suppl.*, 1941, **123**, 164.

[14] Scherf, D., *Z. f. d. ges. exp. Med.*, 1929, **65**, 222.

17042

Cortical Projection of Proprioception in the Cat and Monkey.*

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In spite of the abundant evidence of the importance of proprioception for the regulation of movements in general and those elicited by stimulation of the motor cortex in particular[1-3] little is known about the action of proprioceptive impulses on the cortex.†

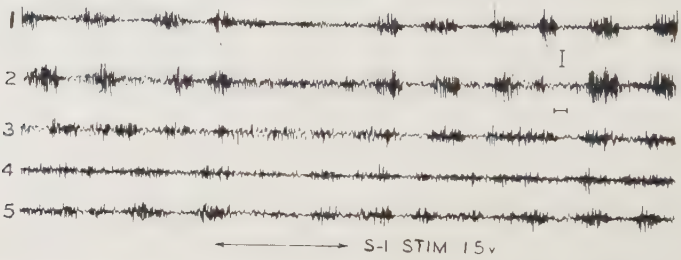
* Aided by a grant from the National Foundation for Infantile Paralysis.

[1] Gellhorn, E., *Brain*, 1948, **71**, 26.

[2] Gellhorn, E., *Brain*, in press. Presented at the Minneapolis meeting of the American Physiological Society, September 1948.

[3] Hyde, J., and Gellhorn, E., *Am. J. Physiol.*, 1949.

FIG 1



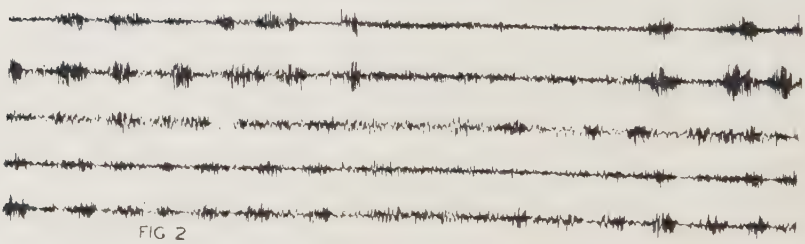


FIG 2

S-1 STIM. 1.9v

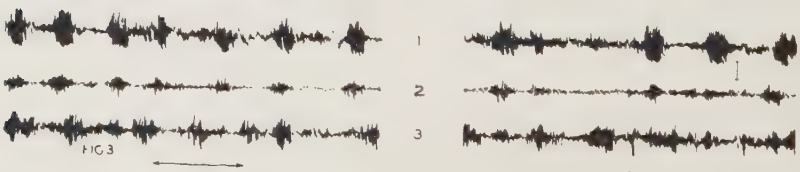
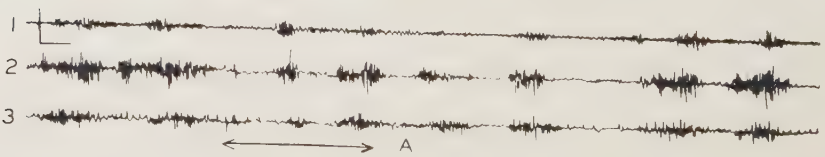


FIG 3



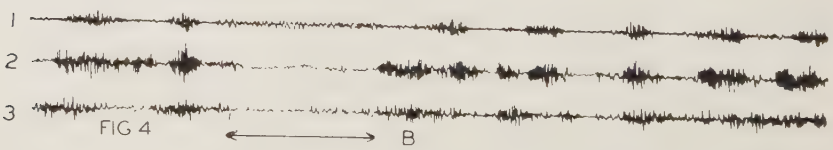


FIG 4

B

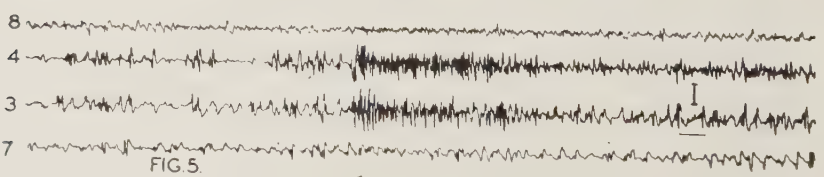


FIG. 5.

FIG. 1. Effect of passive extension of r. hindleg (knee extended to 160°, ankle to plantar flexion of 140°, duration of movement indicated by arrow) on the corticogram of the cat. 1, r, sensorimotor area; 2, l. sensorimotor area; 3, l. temporal region; 4, l. occipital region; 5, l. parietal region. Calibration in Fig. 1 to 5 300 microvolts and one second.

FIG. 2. Effect of stimulation of peripheral end of r. first sacral ventral spinal root (S_1) with condenser discharges (1.5 volts, 90 per second, upper part of figure; 1.9 volts, 90 per second, lower part of figure) on the E.C.G. of the cat. No. 1 to 5 as in Fig. 1. Duration of stimulation indicated by arrow.

FIG. 3. Effect of stimulation of r. S_1 (1.5 volts, 90 per second) on the E.C.G. of the cat before and after reinforcement by fixation of stimulated extremity in extension. 1, l. motor cortex, 2, l. sensory cortex (gyrus proreus), 3, l. auditory area. Left figure without fixation; right figure, knee fixated at 150° and ankle at 130°.

FIG. 4. Effect of stimulation of r. S_1 (1.5 volts, 90 per second) on left E.C.G. of the cat. 1, sensorimotor area, 2, parietal cortex, 3, occipital cortex. R. tibialis anterior and gastrocnemius muscles tenotomized. In experiment A tendons without tension. In experiment B tendons under tension by application of 225 g weight to each tendon.

FIG. 5. Effect of stimulation of r. S_1 (1.0 volts, 90 per second) on contralateral cortex of the monkey. The numbers 8, 4, 3, 7 indicate the eye field, motor area, postcentral gyrus, and parietal lobe respectively.

That the motor cortex is involved is suggested by Bard's[5] observation that the contralateral hopping reaction is abolished in the monkey by ablation of the motor cortex. If the postcentral gyrus alone is removed this reaction is lost temporarily (Peele).[6] That the precentral gyrus plays a role in proprioception is further indicated by the fact that stimulation of posterior roots elicits, in addition to potentials in the postcentral gyrus, also changes in area 4 which are not of tactile origin (Woolsey, Chang and Bard).[7] While the present work was in progress Dawson[8] showed by means of EEG records that stretching a contralateral muscle elicited a myoclonic seizure accompanied by distinct potential changes over the central part of the skull corresponding to the contralateral area 4.

It is the purpose of this paper to report experiments which demonstrate the cortical projection of proprioceptive impulses in cat and monkey.

Method and material. The experiments were performed on 35 cats and 8 monkeys (Macaque) anesthetized with 0.45 cc dial-urethane per kilo i.p. The exposure of brain and spinal cord followed standard procedures. Cortical potentials were recorded with an Offner inkwriter after proper amplification as in previous experiments. For eliciting proprioceptive impulses several methods were used: 1. gentle passive movements of one hindleg; 2. stimulation of the peripheral end of a motor root, usually S_1 as previously described by Cooper and Creed.[9] A shielded electrode of special design was used which permitted repetition of this experiment over periods as long as 24 hours; 3. stimulation of the central end of a muscle nerve with threshold currents.

Results. I. The effect of proprioceptive impulses on the cortex of the cat, a. Effect of proprioceptive impulses induced by passive movements. Passive flexion and extension studied in 5 cats were equally effective in causing an excitation of the cortex which was

† The role of proprioception in cortically induced convulsions has been studied by Gellhorn, Hyde, and Gay.[4]

[4] Gellhorn, E., Hyde, J., and Gay, J., *Arch. Internat. Pharmacodyn.*, 1949.

[5] Bard, P., *Harvey Lectures*, 1938, **33**, 143.

[6] Peele, T. L., *J. Neurophysiol.*, 1944, **7**, 269.

[7] Woolsey, C. N., Chang, H. T., and Bard, P., *Fed. Proc.*, 1947, **6**, 230.

[8] Dawson, G. D., *J. Neurol. Neurosurgery Psychiat.*, 1947, **10**, 141.

[9] Cooper, S., and Creed, R. S., *J. Physiol.*, 1927, **62**, 273, and **64**, 199.

characterized by the disappearance of "Dial" potentials and/or an increase in the frequency and amplitude of the background potentials. Frequently the cortical effect appeared only on the contralateral sensori-motor areas; sometimes (Fig. 1) the effect was present in both sensorimotor cortices but greater on the contralateral side. Temporal, parietal and occipital areas were consistently negative. Passive movements were repeated at intervals of more than three minutes and it was observed that the excitatory effect on the cortex gradually diminished with each successive movement. After 4 or 5 tests the changes in cortical potentials were no longer obtained.

b. *Proprioceptive impulses induced by ventral spinal root stimulation.* Stimulation of the peripheral end of S_1 which produced a strong contraction of both flexor and extensor muscles of the hindleg with condenser discharges of 1.0 to 1.5 volts (90 per second) was used in 11 cats. Electrocorticograms recorded during such stimulation showed an excitation which was greater on the side contralateral to the limb stimulated and most marked in the sensori-motor area. A stronger stimulus was required to elicit both a muscular contraction and a cortical effect than to evoke a muscular contraction alone.

Fig. 2 shows the effect of 2 different intensities of stimulation applied to S_1. The weaker stimulus produced cortical changes confined to the period of stimulation whereas the stronger stimulus greatly prolonged the excitatory effect. The "Dial" potentials disappeared in the sensori-motor areas and were unaltered in the other projection areas on stimulation of S_1 with 1.5 V, but in response to stimulation with 1.9 V some slight excitation was present outside the sensori-motor area although to a much lesser degree than in the latter. The greater amplitude of the potentials in the contralateral than in the ipsilateral sensori-motor area in both tests suggests more recruitment of neurons in the contralateral cortex.

Since it was pointed out in previous investigations that proprioceptive impulses modify cortically induced movements more effectively when the muscle contractions occur under isometric conditions the experiments with stimulation of S_1 were repeated under conditions of fixation of joints. By properly adjusting its intensity a stimulus was used which although inducing a contraction of the muscles failed to alter cortical potentials. If, however, the same stimulus was repeated while the leg was fixated in extension the "Dial" potentials disappeared in the contralateral cortex (Fig. 3). Similar results were seen with fixation in flexion.

These experiments showed that proprioceptive impulses originating in nearly isometrically contracting muscles were particularly effective in altering cortical potentials. As an illustration of primary involvement of muscle tension Fig. 4 shows that S_1 stimulation of tenotomized muscles was ineffective on the cortex while the same stimulus applied when the muscles were kept stretched with a load elicited marked potential changes in the contralateral cortex.

c. *Cortical projection area of proprioception in the cat.* The experiments reported in the preceding paragraphs have already indicated that proprioceptive impulses are projected to the cerebral cortex and involve primarily the contralateral sensori-motor cortex. Even in the recordings in which several diverse areas of the cortex were excited, the potential changes decreased with increasing distance from the sensori-motor cortex. These findings led to a more detailed study of the projection of proprioception to the cerebral cortex (11 cats) based on stimulation of S_1.

A composite map of the contralateral projection area of proprioception is shown in Fig. 6. Each point on the map was verified in 2 or more animals. The principal area excited by proprioceptive discharges was the contralateral sensori-motor cortex while temporal, parietal and occipital areas were unchanged. A few points believed to be located in areas 2s and 19s appeared to be excited.

In contradistinction to this group it was seen in several animals (degree of anesthesia ?) that the cortical response to proprio-

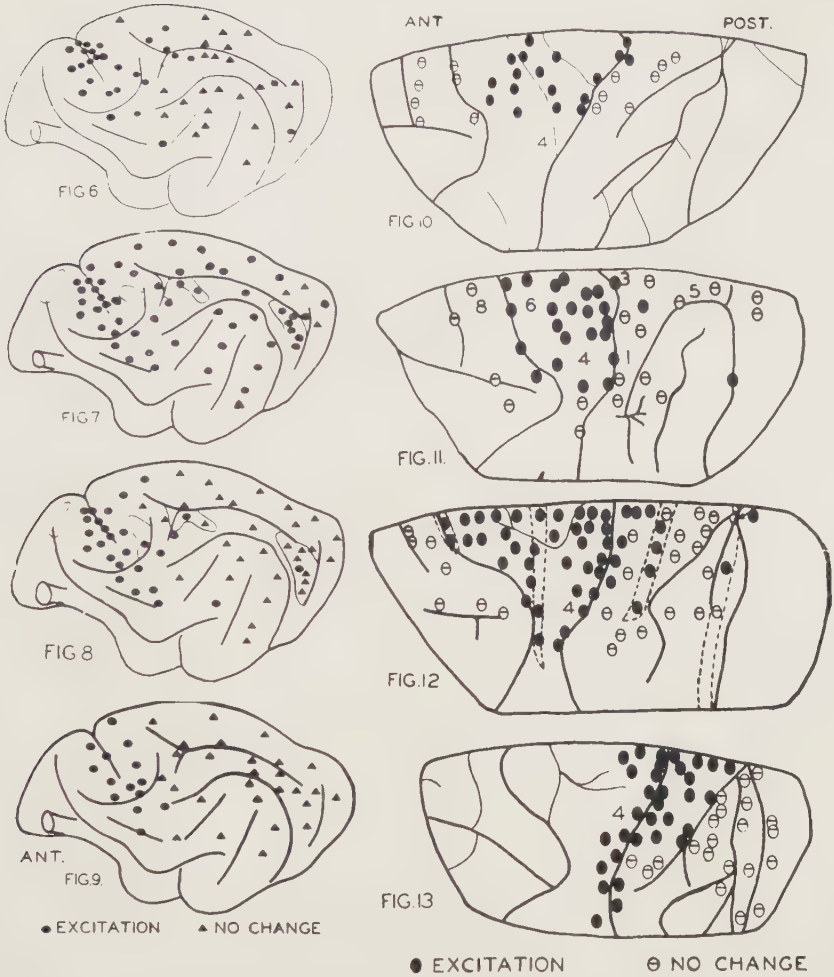


FIG. 6. Projection area of proprioception in the contralateral cerebral cortex of the cat determined by ventral spinal root stimulation or passive movements of extremities in several animals. Oval symbols represent points of excitation, triangles indicate no change. Each point was verified in more than one experiment and more than one animal.

FIG. 7 and 8. Area of projection of proprioception following contralateral stimulation of S_1 in the same cat. In Fig. 7 all points showing disappearance of "Dial" potentials on stimulation are marked positive whereas in Fig. 8 only those points showing in addition increased background activity are indicated by oval symbols.

FIG. 9. Projection of proprioception in the cerebral cortex of the cat determined by stimulation of a nerve from the contralateral gastrocnemius muscle.

FIG. 10 to 13. Projection area of proprioception in the contralateral cerebral cortex of four monkeys determined by ventral spinal root stimulation (S_1).

ception tended to be a diffuse phenomenon. Thus, in the experiment shown in Fig. 7 the entire cortex was responsive except the occipital and temporal poles. However, a careful examination of the records showed that the degree of excitation was quite different in different parts of the cortex. If a distinction is made between reactions consisting of temporary disappearance of dial potentials only and those responses in which in addition the background potentials were markedly increased, it can be shown that the sensorimotor cortex is more strongly affected. In Fig. 8 a map of the same cortex is represented as

in Fig. 7 but only those points are marked as positive which showed increased background activity in addition to the loss of "Dial" potentials (asynchrony plus recruitment. cf. Gellhorn).[10] This figure shows a clear relation of proprioceptive impulses to the sensori-motor cortex. Again two points believed to be located in areas 2s and 19s were positive although surrounded by an unresponsive region.

Proprioceptive projection was also studied by stimulating the central end of the nerve supplying the medial head of the gastrocnemius muscle. At 1.0V a small ipsilateral reflex contraction occurred but no change in cortical potentials. Stimuli of 1.5 or 1.9V resulted in excitation of the contralateral sensori-motor cortex whereas 2.3V caused bilateral excitation of all cortical areas. For mapping purposes the weaker stimulus (1.5V) was used. The effect (Fig. 9) resembled the maps shown in Fig. 6 and 8.

II. *The effect of proprioceptive impulses on the cortex of the monkey.* a. *Proprioceptive impulses induced by ventral spinal root stimulation.* The effects of ventral spinal root stimulation on the cerebral cortex of the monkey closely resemble those seen in the cat. When a subthreshold stimulus (contraction of limb muscles without cortical change) was employed, fixation of the limb in a position of flexion or extension was usually successful in augmenting proprioceptive impulses which led to an excitation of the cortex. The most marked excitation was observed in the contralateral cortex and consisted of a great increase in frequency and amplitude of the potentials in area 4 and the closely adjacent part of the postcentral gyrus while the potentials in area 8 and 7 remain unchanged (Fig. 5). The degree of specificity of the cortical projection area is also indicated by the fact that the various parts of area 4 were unequally affected by stimulation of S_1, the change being the greatest in the medial (hindleg) area and progressively diminishing with increasing laterality.

Experiments on passive flexion or extension showed slight effects on cortical potentials restricted to the contralateral area 4.

b. *Cortical projection area of proprioception in the monkey.* The projection pattern of proprioception was much more specific in the monkey than in the cat. Excitation of the cortex induced by either passive movements, spinal root (S_1) or afferent muscle nerve stimulation was confined to the precentral motor area (area 4 and 6) and to a much lesser extent to adjacent portions of the postcentral sensory cortex (Fig. 10 and 11), In two animals (Fig. 12 and 13) the excitation extended somewhat more posteriorly in the postcentral sensory cortex to include areas 2s in one animal and area 2s and 5 in the other animal. Points anterior to area 6 and posterior to area 5 and those located in the central portion of the temporal lobe were consistently negative (no excitation). None of the monkeys showed a widespread excitation of the cortex of the type seen in the cat when the same ventral spinal root was stimulated.

The degree of excitation induced by proprioceptive impulses was greater on the contralateral motor cortex. The excitation of the ipsilateral hindleg area was very slight on stimulation of S_1 and was of the same order of magnitude as that found in the face area of the contralateral cortex.

Discussion. The uniform results obtained with passive movements, stimulation of the peripheral end of a sectioned motor root, and of the low threshold fibers of a muscle nerve known to consist of proprioceptive nerves indicate that proprioceptive nerve fibers were stimulated. Tactile receptors are not involved since in "Dial" anesthesia methods used in this paper failed to show any changes in the corticogram on tactile stimulation. Neither do nociceptive impulses play any role in these results since the effect of the above procedures is regularly eliminated by ipsilateral hemisection of the spinal cord‡ whereas the widespread cortical action of stimulation of a posterior root persists after this operation.

The effect of proprioceptive impulses on the cortex of the cat was found to be more diffuse than in the monkey. Whether this

[10] Gellhorn, E., and Ballin, H. M., *Am. J. Physiol.*, 1946, **146**, 630.

indicates a species difference or rather a different reaction due to different degrees of anesthesia is impossible to decide at present. The diffuse action noted on the cortex of the cat seems to be akin to a general awakening effect. This is interesting inasmuch as the relation of proprioceptive impulses to the state of wakefulness is well established (Kleitman).[11]

It is worthy of note that in some instances proprioceptive impulses elicited an excitation of cortical suppressor areas. Dick, Bosma and Gellhorn[12] showed recently that stretching of contractured muscles caused a temporary suppression of cortical potentials and of the responsiveness of the motor cortex to electrical stimulation.

Previous experiments have established the fact that cortically induced movements are greatly modified by proprioceptive impulses particularly if the latter are intensified by fixation of joints so that muscle contractions proceed with minimal shortening. The present work gives evidence that proprioceptive impulses alter cortical activity and that the core of this effect resides in the contralateral motor cortex. These impulses are obviously necessary for the performance of cortical proprioceptive reflexes such as the hopping reaction (Bard). However, whether the cortical projection of proprioceptive impulses is responsible for the modification of cortically induced movements is uncertain. Unpublished experiments by Loofbourrow and Gellhorn have shown that spinal nociceptive reflexes are altered in a very similar manner under conditions of proprioceptive reinforcement. Even the myotatic reflex excited by stretch in a given muscle is not restricted to this muscle but extends its effect to other muscles which appear in a similar functional grouping as seen under conditions of stimulation of the motor cortex.[13] It is therefore quite probable that proprioceptive impulses modify cortically induced activity by altering the reactivity of spinal and not that of cortical neurons and this interpretation may be valid for the previously reported effects of nociceptive impulses on cortically induced movements (Gellhorn and Thompson).[14]

It is believed that the most important cortical function of proprioceptive impulses has to do with the initiation of voluntary movements. The fact that a deafferented extremity becomes useless in higher animals although no essential change occurs in the effect of electrical stimulation of the motor cortex (Sherrington,[15,16] Hyde and Gellhorn[3]) suggests an action of proprioceptive impulses on the motor cortex. The experiments presented in this paper have provided this evidence.

Summary. The effect of proprioceptive impulses on the corticogram was studied in cat and rhesus monkey by 1. passive flexion and extension of an extremity, 2. stimulation of the peripheral end of a ventral spinal root with condenser discharges, 3. stimulation of the central end of a muscle nerve. Excitation was indicated by disappearance of "Dial" potentials and/or increase in amplitude and frequency of background potentials. That tension receptors were primarily involved was shown by the fact that a stimulus of S_1, subthreshold as far as the cortical effect was concerned, became effective if the muscles contracted isometrically or under load.

The principal area of the cortex which was excited by proprioceptive impulses was the sensori-motor area in the cat and the precentral motor cortex in the monkey. In several cats the excitation induced by proprioception was spread diffusely throughout the contralateral hemisphere except for the extreme poles, but the excitation was greatest in the

‡ Unpublished observations.

[11] Kleitman, N., Sleep and Wakefulness, Chicago, 1939.

[12] Dick, C., Bosma, J., and Gellhorn, E., *Arch. Internat. Pharmacodyn.*, 1949.

[13] Loofbourrow, G. N., and Gellhorn, E., *Am. J. Physiol.*, 1948, **154**, 433.

[14] Gellhorn, E., and Thompson, L., *Am. J. Physiol.*, 1944, **142**, 231.

[15] Mott, F. W., and Sherrington, C. S., *Proc. Roy. Soc.*, 1895, **57**, 481.

[16] Sherrington, C. S., *Philos. Trans.*, 1893, **184** B, 641.

sensori-motor area. In the monkey the cortical effect was more restricted and appeared in the precentral motor cortex (area 4 and 6) and, to a lesser extent, in the adjacent part of the postcentral sensory cortex. When proprioceptive impulses were elicited in a hindleg of a monkey the area of greatest change of potentials was in a hindleg point of the contralateral motor cortex. Lesser degrees of excitation were seen in arm and face area and also in the ipsilateral leg area.

It is suggested that proprioceptive impulses to area 4 are involved in voluntary movements. Their absence accounts for the loss of voluntary movements of the deafferented limb in the monkey although no change occurs in its responsiveness to electrical stimulation of the motor cortex.

17043 P

Effect of Curare on Autonomic Reflexes.*

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The current trend to utilize curare for muscular relaxation during surgical anesthesia has led to observations disclosing a depressor effect of curare for certain autonomic reflexes.

a. Celiac plexus reflex. Pressure stimulation at the celiac plexus during surgical manipulation may result in reflexogenic changes consisting of a fall in the systolic blood pressure, a marked diminution in pulse pressure, little or no change in pulse rate, and rigidity of abdominal muscles.[1,2] Administration of therapeutic doses of d-tubocurarine to 12 patients who presented such a syndrome during upper abdominal surgery, not only provided adequate muscular relaxation but also resulted in a return in the arterial blood pressure and in the pulse pressure to normal which were then sustained.

Experimental investigations in a series of 8 dogs corroborated the clinical observations. The arterial hypotension with marked decrease in pulse pressure produced by compression of the celiac ganglion were not obtained after the intravenous administration of d-tubocurarine (2 units per kg).

b. Carotid sinus reflex. The results observed following the intravenous injection of d-tubocurarine during carotid sinus stimulation showed similar effects.[3]

Two patients having radical neck dissection manifested characteristic carotid sinus reflex activity with severe arterial hypotension and significant bradycardia. The intravenous administration of 40 units of d-tubocurarine resulted in a restitution of the arterial blood pressure and the pulse rate within 3 minutes.

Experimental investigations in a series of 8 dogs showed similar consistent effects. Upon pulling a tape applied about the carotid bulb during pentothal sodium anesthesia there followed arterial hypotension and bradycardia. After the intravenous administration of d-tubocurarine (2 units per kg) repeated similar stimulation of the carotid sinus failed to elicit any cardiocirculatory disturbance.

* Published with permission of the Chief Medical Director, Department of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or conclusions drawn by the authors.

1 Burstein, C. L., and Rovenstine, E. A., *Arch. Surg.*, 1939, **35**, 599.

2 Burstein, C. L., and Rovenstine, E. A., *Curr. Res. Anesth. and Analg.*, 1938, **17**, 134.

3 Heymans, C., Bouckaret, J. J., and Regniers, P., Le sinus Carotidien, G. Doin et Cie, Paris, 1933.

c. Vagal reflex. In 1857 Claude Bernard showed that the bradycardia or asystole produced by stimulation of the vagus nerve could no longer be obtained after a dog had been curarized.[4] Present clinical and experimental studies have corroborated this long neglected observation.

Faradic stimulation of the vagus nerve low in the neck (10 dogs) during general anesthesia with ether or pentothal sodium caused arterial hypotension as well as bradycardia. The same stimulation after the intravenous injection of d-tubocurarine (2 units per kg) did not cause hypotension or bradycardia.

Clinically, these reflexes of circulatory depression during intrathoracic surgery due to vagal stimulation have been corrected by administration of d-tubocurarine.

Conclusion. The intravenous administration of a dose of d-tubocurarine sufficient to produce intercostal paralysis may abolish or alleviate certain autonomic reflexes that may be encountered during stimulation of autonomic pathways. Cardiocirculatory disturbances incident to stimulation of the celiac plexus, the carotid sinus, and the vagus nerve, may be so treated.

[4] Bernard, Claude, Legons Sur les Effets des Substances Toxigues et Medicamenteures (May 30, 1856), J. B. Bailtiere et Fils, Paris, 1883, 2nd edition, p. 348.

17044

Influence of Estradiol on Alkaline Phosphatase Activity in the Genital Tract of the Rat.

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From the Rice Institute, Houston, Texas.

That alkaline phosphatase may play some integral part in the physiological changes occurring in the genital tract during the normal reproductive cycle and during pregnancy was first suggested by the work of Wislocki and Dempsey.[1] More recently the influence of steroids on alkaline phosphatase distribution in the genital tract of the mouse has been studied by Atkinson and Elftman,[2] and by Jenner,[3] who found that estradiol caused increased phosphatase activity in the circular musculature and in the epithelium of the glands and of the lining of the uterus and vagina of castrated animals.

The work reported here concerns the effect of estradiol on the alkaline phosphatase activity in the genital tract of the rat with special emphasis on its activity in the endometrium of the cervix.

Methods and results. Forty-two albino rats were used in these experiments. Estradiol dissolved in sesame oil, was injected subcutaneously in doses ranging from a single injection of 0.2 μg to 7 daily injections of 0.4 μg. At autopsy, the genital tract, including the vagina, cervix, and uterus, was removed and prepared after the method of Gomori[4] for histochemical determination of alkaline phosphatase. Table I summarizes experimental observations.

Notes on table. A series of 8 female rats, given 2 to 3 weeks rest after removal of ovaries, served as controls. The least amount of phosphatase activity found in any one place in the genital tract of these animals has been assigned a value of one. The amount of enzymic activity found in other locations in the same animals and in treated animals is expressed in multiple factors of this unit.

[1] Wislocki, G. B., and Dempsey, E. W., *Am. J. Anat.*, 1945, **42**, 23.

[2] Atkinson, W. B., and Elftman, H., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 148.

[3] Jenner, R., *Nature*, 1947, **159**, 578.

[4] Gomori, G. J., *Cell. and Comp. Physiol.*, 1941, **17**, 71.

TABLE I.
Effect of Estradiol on Phosphatase Activity.

Group	No. rats	Treatment	Uterine horns			Cervix			Vagina	
			Epithelium	Stroma of endometrium	Muscular layer of myometrium	Epithelium	Stroma	Myometrium	Epithelium	Stroma
I	8	Non-injected castrated females	1	0	0	2	0	0	2	0
II	9	Castrated adult ♀, inj. with 0.4 μg estradiol daily, 4-7 days	2	1-2	1	20	2	1	10	1
III	8	Non-inj. normal adult ♀, various stages of cycle	(Only cervix studied)			Metestrous 10 Proestrous 15	2	1	—	—
IV	10	Castrates and								
	7	Immature animals given single inj. 0.2 to 0.4 μg estradiol	,,	,,	,,	5 (after 24 hrs)	—	—	—	—

Phosphatase activity which was localized in capillary walls is not included in this report. The cervix, in this study, is considered to begin in that part of the genital tract in which there is a sudden transition from columnar epithelium to stratified epithelium lining the lumen.[5]

Group I. Non-injected castrated females. In the endometrial epithelium of the controls, the enzyme was spread evenly throughout the cytoplasm of the cell.

Group II. Injected castrated females. In the endometrial epithelium of the uterine horns, the enzyme was localized only at the free end of the cells. In the endometrial stroma, minor amounts were found in the glandular epithelium, and further deposits were noted in the connective tissue cells. Only the circular layer of the myometrium contained any of the phosphatase.

The most marked increase in alkaline phosphatase activity was found in the cervix. On a relative basis, due to the extremely dense precipitate found in the epithelial cells, the endometrial epithelium has been given a value of 20. This precipitate was found to be distributed throughout the cytoplasm but was negligible or lacking in the nuclei.

Group III. Non-injected normal adult females. Since small amounts of estrogen probably are produced continuously by the mature rat ovary, it is not surprising that alkaline phosphatase activity was found to be high in the genital tract at all stages of the estrous cycle in the normal rat. While the distribution of enzyme was similar to that of the estradiol-injected castrate, there seemed to be a definite cyclic change in the amount of enzyme present. The least activity was noted in metestrous, the most in proestrous.

Group IV. Castrates and immature animals. As a result of a single injection of relatively small amounts of estradiol, there was a detectable increase after 24 hours in alkaline phosphatase activity in the series of both castrate and immature animals. These changes, however, were difficult to determine

[5] Burack, E., Wolfe, J. M., Lansing, W., and Wright, A. M., *Cancer Research*, 1941, **1**, 227.

quantitatively and are a poor criterion for estrogen activity.

Discussion. These studies confirm previous observations that injections of estrogen stimulated increased alkaline phosphatase activity in certain tissues associated with reproduction in rodents.[1,3-6] They also call attention to one of the heaviest deposits of active phosphatase in the rat—that seen in the stratified epithelium lining the lumen of the cervix. In addition, the appearance of enzyme activity after injection of estradiol is noted in the endometrial stroma of the uterus and upper cervix, in contrast to that reported for the mouse.[2] Such activity has, however, been reported for the endometrial stroma of the pregnant sow.[1]

The significance of the relationship of estradiol to alkaline phosphatase in normal reproductive physiology is not known. The enzyme is known to be concerned with calcium metabolism as well as that of phosphorus due to the interrelationship of the two metals in their action in the body. Estradiol is also associated with calcium, causing its removal from some bones while laying it down in others.[7] It is highly possible that it may also have a bearing on the calcium metabolism of certain reproductive tissues. If this be so, it is then conceivable that some of the actions of estradiol may be due to the adjustments of tissue calcium through the relation of the hormone with the enzyme.

Summary. Estradiol causes a spectacular increase in the alkaline phosphatase activity of the genital tract of the rat. This increase is most marked in the stratified epithelium lining the lumen of the cervix, but it is also apparent in the connective tissue cells of the stroma of uterus and uterine cervix and in the circular musculature of the upper part of the genital tract.

In contrast to the stratified epithelium of the cervix and of the vagina, the columnar epithelium of the uterus shows very little phosphatase activity in the presence or absence of estradiol stimulation.

6 Talmage, R. V., *Anat. Rec.*, 1947, **99**, 15.

7 Gardner, W. U., and Pfeiffer, C. A., *Physiol. Rev.*, 1943, **23**, 139.

17045

Uterine Circulation Time in the Pregnant Primate, With the Uterus and Abdomen Intact.

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The determination of uterine circulation rate in the pregnant primate is of considerable importance. Recent investigation has led many authorities to believe that there is a relationship between abnormal uterine circulation and the late toxemic syndrome. The abnormality of circulation is considered to be uterine ischemia but this has never been demonstrated. Page[1] in discussing this theory of toxemia has stated that it is unfortunate that no one has been able to determine uterine circulation times throughout pregnancy. Moreover, Smith and Smith[2] consider that the hormonal changes they find in pre-eclampsia are the result of placental ischemia which is attributable, in turn, to uterine ischemia. The basic cause of this ischemia, which apparently develops late in pregnancy, is another matter. Page[1] lists a group of conditions such as hydromnios, multiple pregnancy, diabetes, etc., in which he feels uterine ischemia is most likely to occur and, indeed, toxemia is frequently found in such conditions

* Fellow, U. S. Public Health Service.

1 Page, E. W., *Obs. and Gyn. Survey*, 1948, **3**, 517.

2 Smith, O. W., and Smith, G., *Am. J. Obs. and Gyn.*, 1949, **56**, 821.

as he mentions. Most often, however, no direct relationship with other pathological processes is observed. Recently a mechanism of physiological growth patterns and shapes in the uterus of pregnant rabbits has been described, aberrations from which would produce myometrial ischemia in the latter part of pregnancy.[3] Comparable changes in the growth and shape of the uterus have been observed to occur in the monkey[4] and in the human.[5] In order to test these findings in the primate and to support or negate the ischemic theory described above, we have evolved a method of studying uterine circulation with the abdomen and uterus intact, at regular intervals throughout pregnancy.

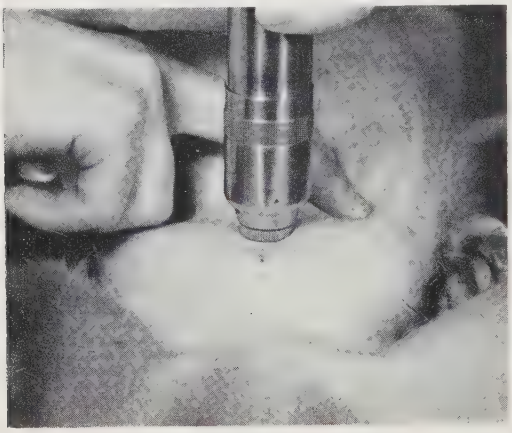

FIG. 1.
The Hypospray shown above will hold up to 1 cc of liquid. Thionine blue is being injected through the abdominal wall of a monkey 60 days pregnant.

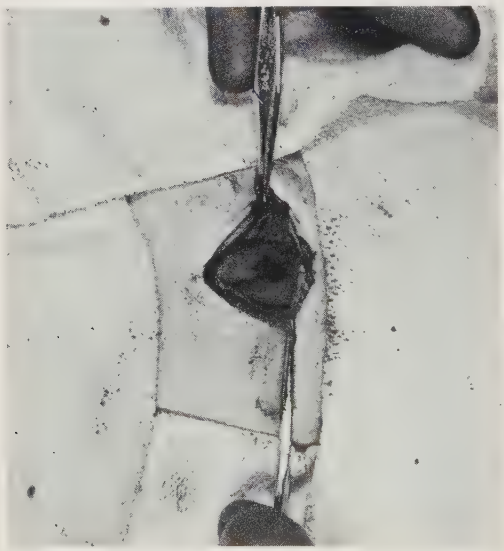

FIG. 2.
At laparotomy uterus of monkey shown in Fig. 1 is seen to contain drug injected through anterior abdominal wall. This is merely a demonstration technic.

Method. The technic described is based upon the ability of uterine muscle to clear a certain amount of dye compared to the ability of the muscle of the anterior abdominal wall to clear an equal amount of the same substance. By use of a new jet injection hypodermic called a "Hypospray" it is possible to propel drugs under the skin almost without pain. The depth of penetration depends upon the strength of the thrust developed which can be controlled by the operator. The Hypospray used in these experiments is modified from the original model described by Hingson[6] in that it is more powerful and can inject a greater amount of drug (1 cc).

The pregnant monkey uterus can be felt abdominally by the fortieth day (duration of gestation—160 days). From this time forward it is possible with a Hypospray to propel a fixed amount of Diodrast into the uterus and an equal amount into the anterior abdominal wall at another injection site. By means of serial x-rays the rate of disappearance of each injection may be observed and charted. The procedure may be performed as often as twice weekly on the same animal without evidence of damage to mother or fetus. Placental separation has not occurred even though there is nearly always an anterior implantation of a secondary placenta in monkeys. The animals gave no sign of a response to pain at the time of injection and such injections have been shown to be practically painless to humans.

A series of preliminary observations has

[3] Reynolds, S. R. M., *Am. J. Obs. and Gyn.*, 1947, **53**, 901.

[4] Gillespie, E. C., Ramsey, E. M., Reynolds, S. R. M., *Am. J. Obs. and Gyn.*, in press.

[5] Gillespie, E. C., manuscript unpublished.

[6] Hingson, R., *Analgesia and Anaesthesia*, 1947, **26**, 221.

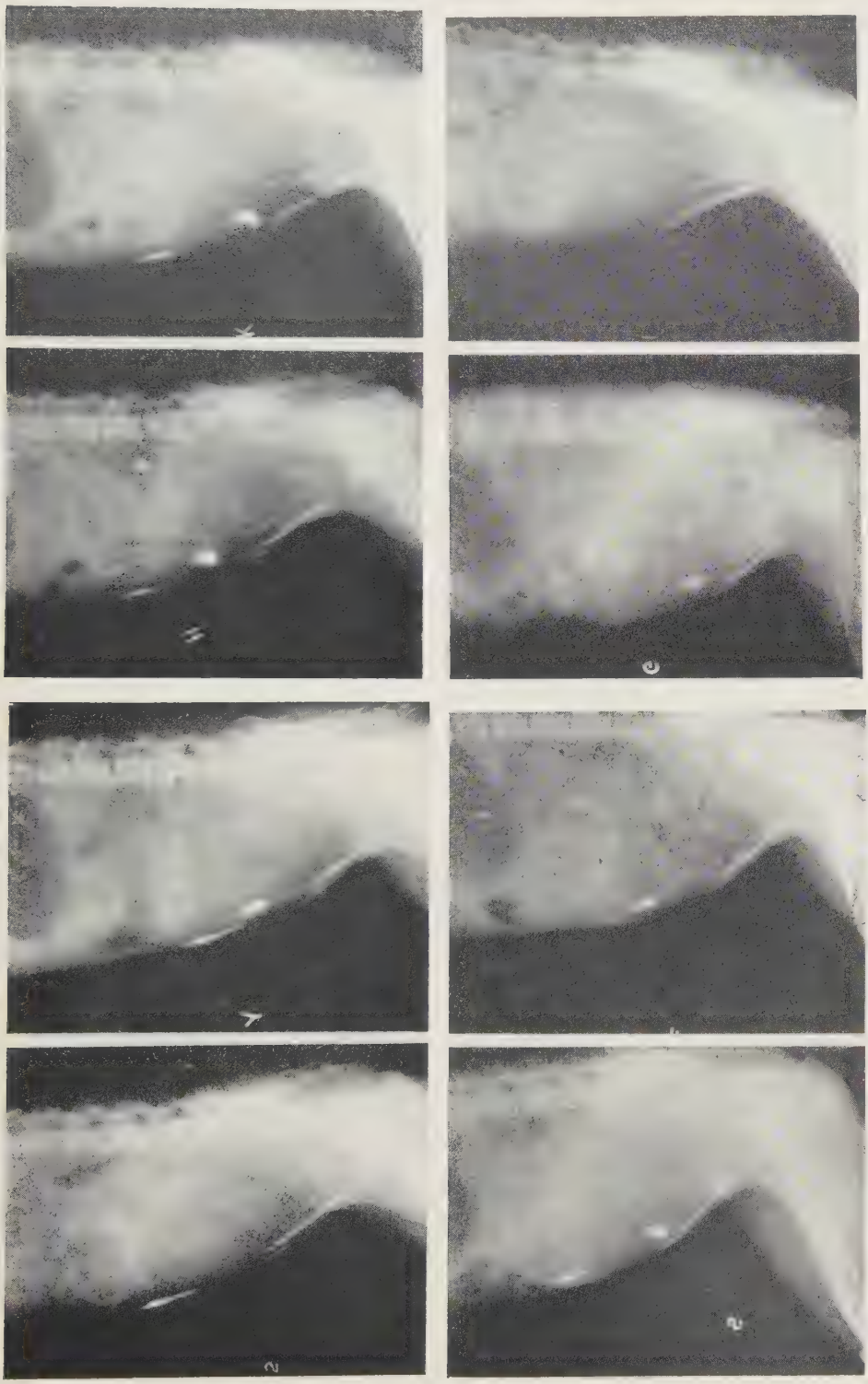

FIG. 3. The x-ray marked (2) was taken immediately following injection of 1 cc of Diodrast into the anterior abdominal wall. The dye can be seen clearly lodged in the rectus muscle just below the level of the fetal head. The x-ray marked (Y) was taken 10 minutes later immediately following the injection of 1 cc of Diodrast into the myometrium. Succeeding x-rays (H, K, S, F, E) were taken at regular intervals until both areas were cleared (1). In the series shown above clearance time for muscle was 25 minutes, and for uterus, 34 minutes. Duration of gestation was 106 days.

been made on monkeys and will be reported later. The technic has been developed in order that it may be adapted to human studies. The absence of pain, and the fre-

quency with which it may be used make it a plan well suited to such a purpose and the investigation is anticipated. The possibility exists, therefore, that uterine clearance times may be done clinically.

Summary. One important reason for measuring uterine circulation throughout pregnancy lies in the possible relationship between ischemia of the gravid uterus and the late toxemias such as pre-eclampsia and eclampsia. We have shown a method of calculating uterine circulation by its ability to clear a radiopaque dye injected by means of a Hypospray into the myometrium. It is shown that the technic can be repeated frequently, is painless and attended by no harmful or uncomfortable sequellae. It is being adapted to human studies.

The authors are indebted to the R. P. Scherer Corp., Detroit, who manufactured the Hypospray used in these experiments, and kindly made it available to us.

17046

Inhibition by Piperidinomethyl-3-benzodioxane (933F) of Epinephrine Vasopressor Blockade Produced by Dibenzyl-β-Chlorethylamine.

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From the Medical Division, Army Chemical Center, Md.

A consideration of the chemical and spatial configuration of compounds which block the pressor action of epinephrine suggests that in all probability dibenzyl-β-chlorethylamine (Dibenamine), ergotoxine, yohimbine, and the phenoxyethylamine type compounds, all block the pressor action of epinephrine by acting on the same mechanism. 933F and ergotoxine have been shown to compete with epinephrine over a wide range of concentrations in isolated physiological systems.[1,2] According to theoretical considerations, ergotoxine, yohimbine, and the phenoxyethylamine type compounds all compete with epinephrine for a receptor of the amine-alcohol configuration in epinephrine. The blocking of this receptor is believed to block the pressor action of epinephrine. Dibenamine is thought to combine chemically with, or near, this same amine-alcohol receptor.

It has been shown by Nickerson and Goodman[3] that the blocking action of Dibenamine is irreversible, in that large doses of epinephrine cannot break through its blocking action and cause a pressor response. This irreversible blocking action persists for 3-5 days after the administration of Dibenamine, by which time more epinephrine receptor is presumed to have been formed. These same authors point out that during the first hour or so after its administration Dibenamine decomposes to a physiologically inactive compound and that all the effects of Dibenamine are due to that fraction which combined with the epinephrine receptors before decomposition occurred. If during this first hour the epinephrine receptors are occupied by a compound which blocks reversibly the action of epinephrine, the Dibenamine should be unable to combine with the receptors and on removal of the reversibly-blocking compound epinephrine should give a normal pressor response. 933F is just such a reversibly-blocking compound which in doses of 5 mg/kg blocks the vasopressor action of epinephrine for 4-5 hours.

Methods. Dogs of either sex under light sodium pentobarbital anesthesia were used for all experiments. The carotid blood pressure was recorded by a mercury manometer. Injections were made into an exposed femoral vein. The 933F was dissolved in a few cc of saline. The Dibenamine was made up 5-20 minutes before use in about 20 cc of saline made strongly acid with hydrochloric

* Captain, Medical Corps.

1 Abdon, N. O., Hammarskjold, S. O., *Acta Physiol. Scand.*, 1940-41, **1**, 85.

2 Gaddum, J. H., *J. Physiol.*, 1926, **61**, 142.

3 Nickerson, M., Goodman, L. S., *Fed. Proc.*, 1948, **7**, 397.

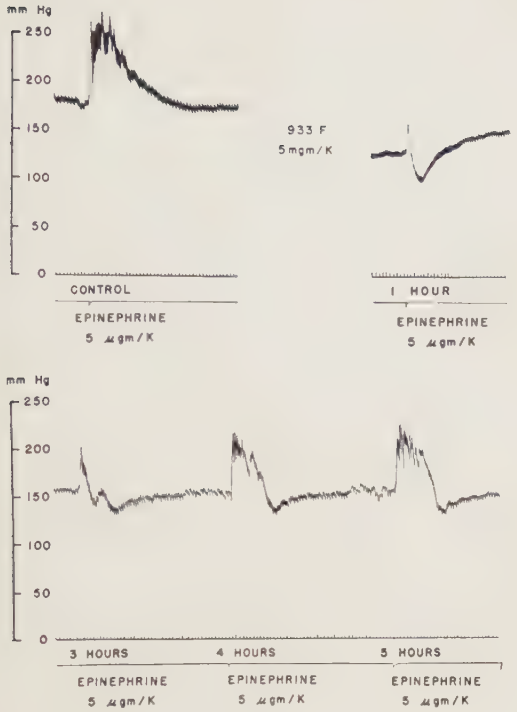

FIG. 1.

Dog, 10.2 kg, female. Sodium pentobarbital anesthesia 30 mg/kg. From top to bottom: carotid blood pressure, 6-second time intervals, injection marker. The 5 blood pressure responses to 5 μg/kg of epinephrine are (reading from left to right): one of 3 control responses obtained before the injection of 933F, and respectively, the responses obtained 1, 3, 4, and 5 hours after the injection of 5 mg/kg of 933F.

acid. The epinephrine used was that available commercially in 1 cc ampules and was diluted 1:10 with saline before injection. All animals were first tested for their response to 3 successive doses of 5 μg per kg of epinephrine. The 933F or Dibenamine was then given and the response of the carotid pressure to 5 μg/kg of epinephrine recorded at hourly intervals thereafter. When both 933F and Dibenamine were injected, the administration of the former drug preceded that of the latter by 3 minutes.

Results. Fig. 1 is a record of the tracings obtained with 5 mg/kg of 933F. By the end of 5 hours the magnitude and duration of the pressor response to 5 μg/kg of epinephrine has returned almost completely to that of the responses obtained before the injection of 933F. Fig. 2 is a record of the tracings obtained with 10 mg/kg of Dibenamine. By the end of 5 hours epinephrine still causes a marked depressor response and there are no signs of return of the initial pressor response. Fig. 3 is a record of the tracings obtained when 5 mg/kg of 933F was given followed in 3 minutes by 10 mg/kg of Dibenamine. The recovery of the pressor response to epinephrine follows essentially the same course as that found after the injection of 933F alone. There is no evidence that Dibenamine exerted any action whatsoever.

In other experiments 5 mg/kg of 933F was given and followed by an infusion of 0.2 mg/kg/min. of 933F for one hour. Recovery of the pressor response to 5 μg/kg of epinephrine was far from complete at the end of 7 hours; however, good pressor responses were obtained from 50 μg/kg of epinephrine. The administration of 20 mg/kg of Dibenamine 3 minutes after the initial dose of 933F de-

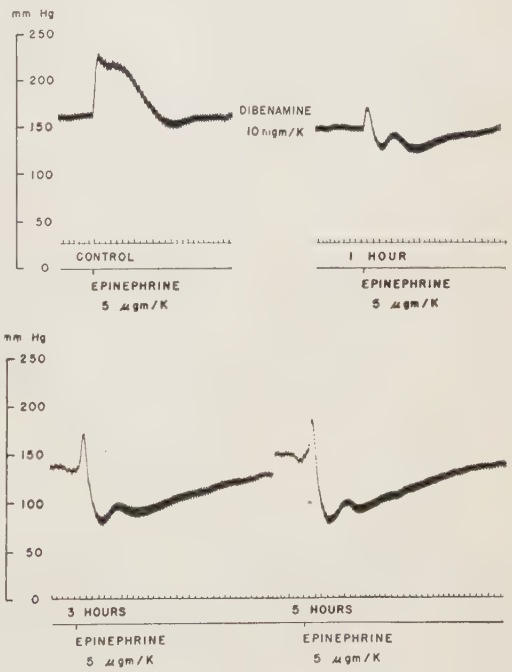

FIG. 2.

Dog, 11.8 kg, male. Sodium pentobarbital anesthesia 30 mg/kg. From top to bottom: carotid blood pressure, 6-second time intervals, injection marker. The 4 blood pressure responses to 5 μg/kg of epinephrine are (reading from left to right): one of 3 control responses obtained before the injection of Dibenamine, and respectively, the responses obtained 1, 3, and 5 hours after the injection of 10 mg/kg of Dibenamine.

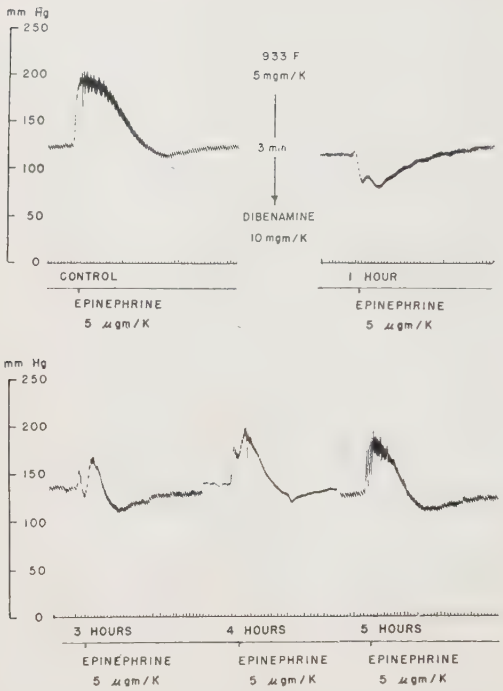

FIG. 3.

Dog, 13.8 kg, female. Sodium pentobarbital anesthesia 30 mg/kg. From top to bottom: carotid blood pressure, 6-second time intervals, injection marker. The 5 blood pressure responses to 5 μg/kg of epinephrine are (reading from left to right): one of 3 control responses obtained before the injection of 933F and Dibenamine, and, respectively, the responses obtained 1, 3, 4, and 5 hours after the injection of 5 mg/kg of 933F followed in 3 minutes by 10 mg/kg of Dibenamine.

pressed only slightly the rate of recovery of the pressor response to epinephrine from that obtained with 933F alone.

Discussion. The fact that 933F prevents Dibenamine from blocking the vasopressor effect of epinephrine may be interpreted in either of two ways. 1. The 933F may aid in the destruction of Dibenamine by either chemically combining with the Dibenamine or enabling something else to do so. 2. The 933F may prevent the Dibenamine from combining with the epinephrine receptors by protecting the receptors. From theoretical considerations and experiments now in progress the latter is considered to be the more likely explanation.

Summary. 5 mg/kg of 933F will prevent 10 mg/kg of Dibenamine from blocking the vasopressor effect of epinephrine in the dog.

The 993F used in these experiments was supplied by Dr. John E. Howard, who obtained it through the courtesy of E. Fourneau.

17047

Contamination of Commercial p-Aminohippuric Acid with p-Aminobenzoic Acid.

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(Introduced by Homer W. Smith.)

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While investigating the oral administration of p-aminohippuric acid (PAH) in man for the purpose of renal clearance measurement, it was noted that different samples of PAH, when administered according to the same routine, gave markedly different concentrations of chromogen in the plasma. PAH was determined by the method of Smith *et al.*[1] in a cadmium sulfate filtrate of plasma.

The plasma concentration of chromogen following the ingestion of 5-6 g of material in divided doses averaged 2.4 mg % (expressed as PAH) in 23 subjects receiving lot No. 12322* while the plasma concentration of chromogen averaged 0.22 mg % in 14 subjects receiving lot No. 236518.* Four tests were then performed on 2 subjects so that

[1] Smith, H. W., Finkelstein, N., Aliminosa, L., Crawford, B., and Graber, M., *J. Clin. Invest.*, 1945, **24**, 388.

* National Aniline Division, Allied Chemical and Dye Corporation.

each received each lot of PAH under identical conditions. Lot No. 12322 gave an average plasma concentration of chromogen of 2.76 mg % in one subject and 3.05 mg % in the second, whereas lot No. 236518 gave an average plasma concentration of 0.112 mg % in the first subject and 0.329 in the second. A third subject, started on lot No. 12322 by mouth attained a plasma concentration of 2.4-3.6 mg %. When he was changed to lot No. 236518, the plasma concentration fell to 0.41 mg %. A 50 to 50 mixture of the 2 lots produced a plasma concentration about midway between the figures produced by the compounds when administered separately.

To rule out the possibility that one compound was being metabolized, a number of experiments were performed on dogs, contrasting parenteral with oral administration. The two lots gave essentially the same plasma concentration when administered parenterally. However, a marked difference between them appeared after oral administration.

In identical experiments, lots No. 12322, No. 236518 and p-aminobenzoic acid (PAB) were compared on oral administration. Again the two lots of PAH produced a marked difference in plasma chromogen. In the dog receiving an equivalent dose of PAB, plasma chromogen concentration successively increased from 3.18 mg % at 10 minutes to 14.6 mg % at 51 minutes, with a slow decline apparent after the latter time. The plasma chromogen values in this dog were as much as 100 times as great as those produced typically by lot No. 236518.

Chemical studies. Intensity of color development. Lot No. 12322 yielded about 5% greater color per unit weight at a wave length of 540 mμ. Absorption curves from 400 to 600 mμ on a 0.1 mg % solution of each lot were identical except for the difference in intensity noted above.

Titration curve. 10 cc aliquots of 100 mg % solution prepared with carbon dioxide-free water were titrated with 0.0107 N barium hydroxide solution and the hydrogen ion concentration determined with the Cambridge pH meter. Lot No. 12322 had about 5% more available acid than lot No. 236518.

Solubility curves. Increasing quantities of each lot were placed in tubes with a measured amount of water and shaken in a constant temperature water bath at 24.6°C until equilibrium was attained. The concentration of acid in the liquid was then determined by titration with barium hydroxide. Lot No. 12322 showed increasing solubility with increasing quantities of excess substance, indicating the presence of a mixture, whereas lot No. 236518 showed a sharply breaking solubility curve with constant solubility in the presence of excess substance, indicating a relatively pure compound.

By methods of approximation it was estimated that impurities present in lot No. 12322 comprised between 17 and 31% by weight.

Infra-red absorption spectra. Infra-red absorption spectra on the above two lots and on a third lot (No. 12738) confidently known to be pure PAH,† showed identical absorption bands with correct characteristics of a p-aminophenyl substitution. M-aminohippuric acid showed a quite different and characteristic absorption spectrum.

The melting point (uncorrected) of lot No. 12322 was 165-169°C, of lot No. 236518, 200-202°C; lot No. 12738, 196-198°C; a 50-50 mixture of lots No. 236518 and 12322 melted at 182-185°C; a mixture of lots No. 236518 and 12738 melted at 186-188°C, and a mixture of lots No. 12322 and 12738 at 159-164°C. The recorded melting point for PAH is 198.5°C,[2] and for PAB, 187°C.[3]

Extraction data. 22 g of lot No. 12322 were extracted with ether and yielded 15 g of PAH, 4 g of PAB, and 1 g of unresolved residue. The PAB was identified as such by its melting point (183-185°C) and the fact that the melting point was not depressed by mixture with authentic PAB. The nitrogen content was found to be 10.2-10.4°C (theoretical for PAB is 10.4). The acid equivalent was 135 (theoretical 137). The preparation

† Kindly supplied to us by the National Aniline Division, Allied Chemical and Dye Corporation.

2 Cohen, P. P., and McGilvery, R. W., *J. Biol. Chem.*, 1946, **166**, 261.

3 Beilstein, *Handbuch der Organischen Chemie*, 1931, **14**, 419.

had the typical crystalline form of PAB.

The PAH extracted above melted at 198-200°C, contained 14.1-14.4% nitrogen (theoretical is 14.4), and had an acid equivalent weight of 189-195 (theoretical is 194.2).

Purification of an aqueous iso-electric solution can be effected by extracting with ether and evaporating the ether to dryness. The residue *dissolved in hot water* yields crystalline PAB on cooling. The ether-extracted aqueous solution can be crystallized directly to give PAH which may be recrystallized from absolute alcohol.

Discussion. We conclude from the above data that lot No. 236518 is essentially pure PAH, whereas lot No. 12322 contains approximately 23% of PAB. Incidentally, it is demonstrated that PAB is rapidly absorbed from the intestinal tract from dog and man, whereas PAH is slowly and poorly absorbed.

PAH is made commercially by the conjugation of p-nitrobenzoyl chloride with glycine, the nitro group being subsequently reduced. Failure to effect complete conjugation, or hydrolysis during reduction, will yield PAB. Since the clearance of PAB is less than the creatinine clearance in dogs,[1] the presence of a significant quantity of this substance in PAH would introduce a large error into the determinations of the renal plasma flow. The Medical Division of Sharp and Dohme, who prepared ampouled PAH for clinical investigation, report that lot No. 12322, which we find to be contaminated with PAB, has never been marketed, and they are now examining all samples for significant contamination by PAB.

Summary. P-aminobenzoic acid is rapidly absorbed from the gastrointestinal tract in dog and man while p-aminohippuric acid is poorly absorbed.

One commercial lot (National Aniline No. 12322) of p-aminohippuric acid was found to be contaminated with 23% of p-aminobenzoic acid.

None of the contaminated material, to our knowledge, has been marketed in ampouled form for clinical investigation and an adequate control is now being maintained on the purity of clinical material.

We are indebted to Joanne Baker Schreiner for technical assistance and to Dr. Conrad Dobriner of the Sloan-Kettering Institute, New York City, for determining the infra-red absorption spectrum of the several compounds studied.

We are also indebted to Dr. R. Keith Cannan for his assistance in chemical purification, and to Sharp and Dohme for parallel chemical studies and for rechecking their file samples of their ampouled material.

17048 P

Recovery of Isotopic Succinate from Urine of Rats Administered Isotopic Acetate.

JUI SHUAN LEE AND NATHAN LIFSON.

From the Department of Physiology, University of Minnesota Medical School, Minneapolis.

To demonstrate that a given pathway is involved in the metabolism of an administered isotopic compound, it would appear essential to determine the isotopic composition of the assumed intermediates in the pathway. Evidence is accumulating that the Krebs or tricarboxylic acid cycle, developed mainly on the basis of *in vitro* evidence, actually operates in the intact animal, and that this scheme participates in the metabolism of acetate.[1-4] In the present investigation it was found that after the administration of isotopic acetate

[1] Krebs, H. A., and Johnson, W. A., *Biochem. J.*, 1938, **32**, 113.

[2] Lorber, V., Lifson, N., and Wood, H. G., *J. Biol. Chem.*, 1945, **161**, 411.

[3] Lifson, N., Lorber, V., Sakami, W., and Wood, H. G., *J. Biol. Chem.*, 1948, **176**, 1263.

[4] Rittenberg, D., and Bloch, K., *J. Biol. Chem.*, 1945, **157**, 749.

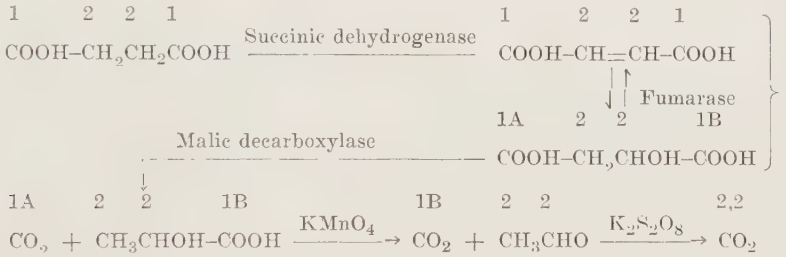
to rats, labeled carbon is found in succinate recovered from the urine, and that the location of isotope in this intermediate of the tricarboxylic acid cycle corresponds to the distribution predicted from the reactions of the cycle.

Methods. Two ml of 0.5 M sodium malonate per 100 g of body weight was administered subcutaneously and 1.10 mM of $CH_3C^{13}OONa$ (6.10 atom % excess C^{13} in the carboxyl group) per 100 g of body weight was fed by stomach tube to fasted rats weighing 120 - 200 g. The malonate was injected to augment urinary succinate excretion.[1] The acetate dose was repeated at the end of approximately 8 and 16 hours. The urine was collected in 20% H_2SO_4 for 24 hours, except during periods when respiratory carbon dioxide was being sampled.

The urine was extracted with ether in the presence of sodium bisulfite in order to bind keto acids. The ether extract was distilled with steam and the residue oxidized with acid permanganate. Succinic acid was extracted with ether from the oxidation mixture and purified by repeated silver precipitations.[5] The melting point of the white crystalline product was 180° - 185° and that of the Eastman Kodak product 185° - 187°.

The succinic acid recovered from the urine was converted into a mixture of fumaric acid and malic acid with succinic dehydrogenase prepared from pigeon breast muscle.[6] Fumaric and malic acid were extracted with ether and subjected to the action of an acetone powder of *Lactobacillus arabinosus* prepared by Ochoa's procedures[7] with the exception that fumaric acid instead of malic acid was used in the culture medium. The former proved to be more effective in developing fumarase as well as malic decarboxylase activity. This enzyme preparation converts quantitatively the mixture of fumarate and malate into lactic acid and CO_2.

The lactic acid thus formed was oxidized by permanganate into aldehyde and CO_2. The aldehyde was oxidized by potassium persulfate to CO_2.[8] The overall degradation scheme may be summarized as follows:



At each step, the yields of degradation products from urinary succinate compared satisfactorily with theoretical expectations and with those from authentic succinate.

Results and discussion. From the data in Table I, it will be noted that after the administration of $CH_3C^{13}OOH$, $C^{13}OOHCH_2$-$CH_2C^{13}OOH$ is recovered from the urine. This is the result predicted from metabolism of acetate via the tricarboxylic acid cycle, by condensation with oxalacetate.[9] Since appreciable amounts of C^{13} also appeared in the respiratory CO_2 (.02 - .55 atom% excess, depending on the time interval between sampling and acetate feeding), and since CO_2 fixation would also be anticipated to yield carboxyl labeled succinate,[9] it is not possible on the basis of these results alone to evaluate the extent to which the latter mechanism accounts for the observations. The small but significant difference in C^{13} concentration between the 2 carboxyl-carbons (fractions 1A and 1B), which theoretically should be equal because

5 Wood, H. G., Werkman, C. H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, 1942, **142**, 31.

6 Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric Techniques, 1945, p. 144, Burgess Publishing Co.

7 Ochoa, S., personal communications; Korkes, S., and Ochoa, S., *J. Biol. Chem.*, 1948, **176**, 463.

8 Osborn, O. L., and Werkman, C. H., *Ind. and Eng. Chem. Anal. Ed.*, 1932, **4**, 421.

9 Wood, H. G., *Physiol. Rev.*, 1946, **26**, 198.

TABLE I.
Distribution of C^{13} in Urinary Succinic Acid After Administration of Carboxyl Labeled Acetate to Rats. C^{13} Values Are in Atom % Excess.

Exp. No.	Rat No.	Labeled acetate fed mM/100 g	C^{13} in whole succinic acid molecule	C^{13} in degradation fractions: Carboxyl carbons 1A	Carboxyl carbons 1B	Methylene carbons 2,2
I	1	1.1	0.15			
II	2	3.4	0.14			
III	3-8	3.4	0.10	0.21	0.17	0,00

succinate and fumarate are both symmetrical molecules, is interpreted as due to the oxidation of a certain amount of extraneous carbon by permanganate, but other explanations are possible.[10]

These experiments are being extended.

Summary. After the administration of malonate plus carboxyl-labeled acetate to rats, there has been recovered from the urine carboxyl-labeled succinate, an intermediate of the tricarboxylic acid cycle. This result is consistent with and additional evidence for the metabolism of acetate via the tricarboxylic acid cycle in the intact mammal.

We wish to thank Mr. Richard E. Halsted and Mrs. Ruth Boe for performing the isotopic analyses under the supervision of Dr. A. O. C. Nier, Mrs. Shirley L. Michel for valuable technical assistance, and Dr. S. Ochoa for kindly supplying us with cultures of *L. arabinosus*.

[10] Calvin, M., and others. Isotopic Carbon, 1949, p. 192, John Wiley & Sons, Inc.

17049

Simultaneous Fluorocardiography and Recording of Intracardiac Pressure.

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From the Department of Radiology, Beth Israel Hospital, Boston.

Various studies of clinical fluorocardiography have been published in the last three years.[1-5] This method is based on the photoelectric recording of the motion of selected points of the cardiovascular contour as revealed by the fluoroscope.

Although simultaneous recordings of other graphic tracings (electrocardiogram, carotid tracing, phonocardiogram, etc.) have supported the reliability of the fluorocardiogram, an incontrovertible demonstration of the accuracy of the latter has been lacking. For this reason, fluorocardiograms of the right auricle or the right ventricle, simultaneous with tracings of pressure in the corresponding chamber, have been taken in the anesthetized dog.

Materials and methods. A Sanborn electrokymograph and a Sanborn electromanometer were used. The former is a standard apparatus for fluorocardiography; the latter is a new apparatus, based on the strain gauge principle, which permits the recording of variations of pressure with controlled amplification. A Sanborn tri-beam cardiette was employed for the transcription of the tracings so that both tracings were recorded by two separate channels while a third recorded the heart sounds through a stethoscopic micro-

[1] Henny, G. C., and Boone, B. R., *Am. J. Roentgen.*, 1945, **54**, 217.

[2] Luisada, A. A., Fleischner, F. G., and Rappaport, M. E., *Am. Heart J.*, 1948, **35**, 336 and 348.

[3] Luisada, A. A., and Fleischner, F. G., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 436; 1948, **67**, 535; 1948, **69**, 23.

[4] Luisada, G. C., and Fleischner, F. G., *Am. J. Med.*, 1948, **4**, 791.

[5] Luisada, G. C., and Fleischner, F. G., *Acta Card.*, 1948, **3**, 308.

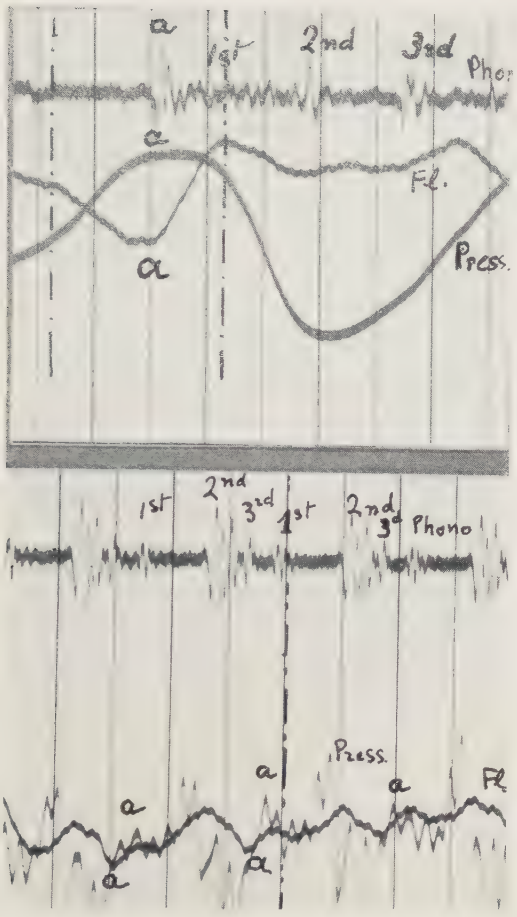

FIG. 1.
Tracings of the right auricle of two dogs. From above: phonocardiogram, fluorocardiogram, pressure tracing (electromanometer).
(A) Rubber catheter.
(B) Woven catheter.

phone applied to the left chest. Pressure tracings were obtained by introducing either a 14 French rubber catheter or a conventional, woven catheter into the right cardiac chambers through the right external jugular vein under fluoroscopic control.

Results. The heart sounds, the fluorocardiogram of the border of the right auricle, and a pressure tracing from the right auricle are recorded in the first figure.

The fluorocardiogram presents a deep negative wave in presystole (contraction of the auricle), a sharp rise at the beginning of systole, and a slower drop during systole. The pressure tracing with the rubber catheter presents a slow rise in early diastole; a more rapid rise in presystole (contraction of the auricle) and a sharp drop in systole. The *presystolic drop* in the fluorocardiogram is simultaneous with the *presystolic rise* of the pressure tracing. The systolic changes, due to the effects of ventricular systole, are revealed by a simultaneous drop in both tracings. The tracing recorded by means of the woven catheter presents multiple vibrations, 2 of which coincide with the presystolic wave of the fluorocardiogram (Fig. 1B).

In the second figure, the heart sounds, a

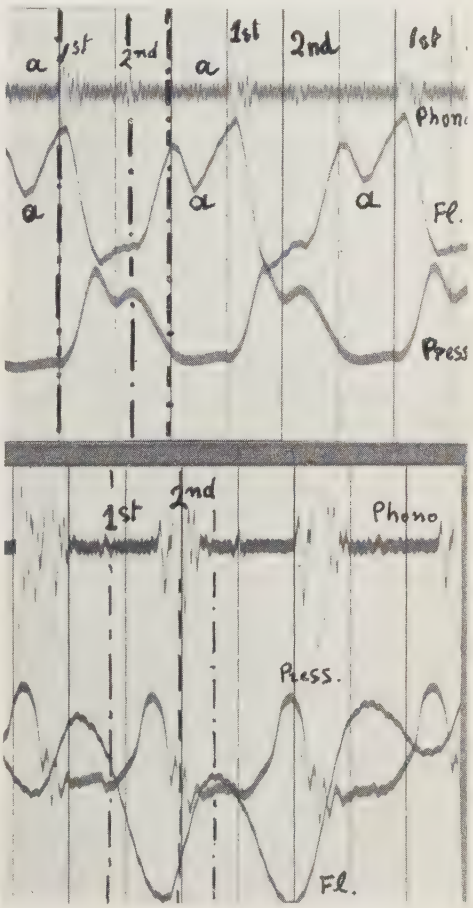

FIG. 2.
Tracings of the right ventricle of 2 dogs. From above: phonocardiogram, fluorocardiogram, pressure tracing.
(A) Rubber catheter.
(B) Woven catheter.

fluorocardiogram of the border of the right ventricle, and a pressure tracing from the right ventricle are recorded. The fluorocardiogram presents a small presystolic negative wave (transmitted from the auricle); a deep *systolic collapse* (ventricular contraction); and a small, early-diastolic rebound. The pressure tracing presents no waves in diastole. Ventricular systole is accompanied by a *systolic plateau* with a higher initial peak.

The temporal relationship between the negative *ventricular wave* of the fluorocardiogram and the *systolic plateau* of the pressure tracing is precisely the same, allowing for a brief lag because of the fact that the ventricular wave of the fluorocardiogram starts with the beginning of ejection.

The tracing, recorded by means of the conventional catheter, presents a more rounded curve of intraventricular pressure (Fig. 2B).

Discussion. This study reveals a precise coincidence between the auricular and ventricular waves of the pressure tracing on the one hand and the fluorocardiographic waves on the other hand. The *positive wave* in the pressure tracing is due to the rise in pressure on contraction of the chamber while the *negative wave* in the fluorocardiogram is due to simultaneous decrease in volume of the chamber. Since the dog's heart shifts horizontally only very slightly within the chest, being more vertical than the human heart, experimental fluorocardiograms are nearly pure volume tracings. Thus a further proof is given of the accuracy of fluorocardiography in recording volume changes of the heart whenever the complicating elements of motion are absent or minimal.

Summary. Simultaneous fluorocardiograms of the right auricle and ventricle and tracings of intracardiac pressure reveal a precise temporal coincidence between the waves of the two records. Therefore, a further proof is given of the accuracy of fluorocardiography.

17050 P

Use of Radiopotassium for Detection of Minute Amounts of Desoxycorticosterone.*

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This communication is a report of a preliminary study on the use of radiopotassium for assay of adrenal cortical hormones. The fact that adrenalectomized rats tend to retain potassium, which may be reversed by the administration of adrenal cortical extracts or pure adrenal cortical steroids, is well known. With the use of radiopotassium as little as 10 μg of desoxycorticosterone may be detected in the adrenalectomized rat.

Animals, methods, materials. Albino rats obtained from Carworth Farms, Inc. were used for these studies. The animals were bilaterally adrenalectomized in one stage under ether anesthesia. Experiments were run 24 hours after adrenalectomy. The diet both before and after the operation consisted of Purina Fox chow without added sodium chloride.

The procedure consisted in the subcutaneous administration of the test material, desoxycorticosterone or desoxycorticosterone acetate,† contained in corn oil. The volume of oil was 0.25 cc. One hour after the administration of the steroid, potassium chloride

* Supported in part by grants from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service, and from Sharp and Dohme, Inc.

† The desoxycorticosterone and desoxycorticosterone acetate were kindly supplied by Ciba Pharmaceutical Products, Inc.

TABLE I.
Influence of Desoxycorticosterone on Excretion of Potassium in Adrenalectomized Male Rats.
(Experiments run 24 hours after adrenalectomy.)

	Control		Experimental				
KCl admin. μg/g B.W.	Mean B.W., g ± S.E., (No.)	Mean K excretion, % ± S.E.	Desoxycorticosterone admin., μg	Mean B.W. g ± S.E. (No.)	Mean K excretion, % ± S.E.	Change, %	t
20	126 ± 4 (11)	6.78 ± 1.30 —	32	126 ± 3 (11)	9.71 ± 2.0 —	+43	1.23
"	126 ± 4 (10)	6.78 ± 1.30 —	500 (Acetate)	126 ± 4 (10)	6.73 ± 1.40 —	+ 0	—
146	126 ± 3 (9)	4.90 ± 0.15 —	32	130 ± 5 (8)	6.34 ± 0.28 —	+29	4.24
"	126 ± 3 (9)	4.90 ± 0.15 —	500	127 ± 3 (10)	7.09 ± 0.28 —	+45	6.76
182	207 ± 6 (12)	2.98 ± 0.33 —	200 (Acetate)	210 ± 8 (12)	4.16 ± 0.24 —	+37	2.22
"	207 ± 6 (12)	2.98 ± 0.33 —	1000 (Acetate)	199 ± 8 (12)	4.51 ± 0.51 —	+51	2.40
280	135 ± 8 (10)	5.81 ± 0.37 —	32	131 ± 6 (10)	8.03 ± 0.39 —	+38	4.11
"	127 ± 3 (11)	5.22 ± 0.51 —	20	128 ± 3 (11)	8.81 ± 0.81 —	+69	3.49
"	131 ± 3 (11)	5.12 ± 0.82 —	20	132 ± 3 (11)	8.99 ± 0.51 —	+76	4.05
"	131 ± 3 (11)	5.12 ± 0.82 —	10	134 ± 2 (10)	9.90 ± 0.93 —	+93	3.72
"	131 ± 3 (11)	5.12 ± 0.82 —	1	125 ± 2 (10)	5.99 ± 0.77 —	+17	0.84

containing radiopotassium (half life 12.8 hours)‡ in the amount of 20 to 280 μg per gram of body weight was injected subcutaneously. The dose of potassium chloride was contained in 2 cc of solution. Urine was collected for 6 hours starting from the time of administration of the potassium chloride solution. The urine was dried and the concentration of radiopotassium in the residue determined as described previously for radiosodium.[1]

The amount of radiopotassium excreted in the urine during the six-hour period was related to the total radiopotassium administered and expressed in per cent. This was done for the results obtained on the experimental animals, as well as for the results obtained on oil injected controls run simultaneously. The significance of the difference in mean percentage excretion of radiopotassium was calculated.

Results. The results of the administration of varying amounts or hormone and potassium chloride to the adrenalectomized male rats are presented in Table I. When 20 μg of potassium chloride per gram of body weight was administered, no significant increment in potassium excretion was found for either 32 μg of desoxycorticosterone or 500 μg of the acetate. In the remaining experiments potassium chloride in the amounts of between 146 to 280 μg per gram of body weight was administered. In each experiment statistically significant increases in potassium excretion were observed. Thus, in one experiment when as little as 10 μg of the steroid was administered, an increase in potassium excretion of 93% was found. The t value was 3.72 indicating a P value of less than 0.01.

Conclusion. Thus far only desoxycorticosterone has been studied with respect to potassium metabolism in the adrenalectomized animal. Under similar conditions it has been previously demonstrated[1] that as little as one microgram produces a significant retention of sodium in the adrenalectomized rat. With radiopotassium 10 micrograms can be detec-

‡ The radiopotassium was supplied by the Monsanto Chemical Co., through the U. S. Atomic Energy Commission.

1 Dorfman, R. I., Potts, A. M., and Feil, M. L., *Endocrinology*, 1947, **41**, 464.

ted. This method affords a convenient way to evaluate the relative activity of various adrenal cortical steroids and adrenotrophic hormone. Further studies are needed to define the method as a quantitative assay for adrenal cortical steroids.

Summary. By the use of radiopotassium in the adrenalectomized male rat, it is possible to detect as little as 10 μg of desoxycorticosterone. The method affords a convenient means of evaluating the relative activities of adrenal cortical steroids, as well as the influence of adrenotrophic hormone on potassium metabolism.

17051

Time of Appearance of Antibodies to Brain in the Human Receiving Anti-Rabies Vaccine.

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Recent reports[1–12] have shown that a disseminated type of encephalomyelitis can be produced in experimental animals by the injection of homologous and heterologous brain tissue, and that the lesions produced resemble the lesions found in the human demyelinating diseases including the encephalomyelitis following anti-rabies vaccination.

This has suggested the possibility that isoimmunization to brain tissue may be the mechanism responsible for the production of these diseases, and, in particular, of the encephalomyelitis following anti-rabies vaccination, since in the latter, the injection of heterologous brain tissue duplicates in many ways the actual experimental procedure which has been followed with the experimental animals.

It seemed of interest, therefore, to determine, in a preliminary study (of 5 cases) whether or not patients receiving anti-rabies vaccine actually develop antibrain antibodies in their sera. The procedure followed consisted of (1) the immunization of rabbits to determine the antigenicity of the brain extract used, and (2) the titration of the sera of patients receiving the anti-rabies vaccine, with this particular antigen.

Materials and methods. I. Preparation of antigens (Lewis[13]):

A. Suspension. The white matter from fresh human brain was dissected free of cortex, connective tissue and blood vessels, cut into thin slices and washed in running water for 30 minutes. It was then ground in a Waring blendor and made into a 30% saline suspension with 0.5% phenol added. This stock solution was used in the immunization of 4 rabbits.

B. Alcoholic extract. The same procedure

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TABLE I.
Complement Fixation Titers Obtained in Rabbits Given Intravenous Injections of Human Brain Mixed with Normal Rabbit Serum.

Rabbit No.	Highest titers obtained			
	8 days	16 days	24 days (Inj. dis.)	40 days
889	1/20	1/40	1/45	1/10
979	1/10	1/30	1/40	1/10
991	1/5	1/20	1/40	1/20
987	1/10	1/30	1/30	1/20

was followed except that after grinding the brain tissue in the Waring blendor, the material was mixed with 10 times its volume of 95% ethyl alcohol and kept in the incubator at 37°C for at least 2 weeks.

II. Immunization of animals (Lewis). The rabbits were given intravenous injections of 1.5 ml of the 1:30 brain suspension every 4 days for 24 days. The suspension was mixed with an equal volume of fresh normal rabbit serum, incubated 2-3 hours at 37°C, and the mixture injected very slowly. The rabbits were bled once every 8 days from the marginal ear vein, the blood allowed to clot, the serum separated by centrifugation and frozen at –30°C until used.

III. Technic of complement fixation. The alcoholic extract alone was used as the antigen, both in the case of the rabbit sera, and in the subsequent fixation with human sera. It was found that a 1:40 dilution of the alcoholic extract in 0.9% saline served adequately without being hemolytic or anticomplementary, and without showing non-specific complement fixation with normal human sera. The amount of antigen per dry weight and in aliquot samples of the extract was checked periodically, and was found to remain relatively constant at 3 mg of solids per ml of the extract. In all instances, 0.1 ml of the 1:40 dilution of the alcoholic extract was used as the antigen; 0.1 ml of the inactivated serum, as well as of each dilution, and two units of guinea pig complement were added and the tubes kept in the refrigerator at 3-4°C overnight. The tubes were then allowed to stand at room temperature for one hour; 1.0 ml of a 2.5% suspension of sensitized sheep red cells added, and the tubes incubated at 37°C for 30 minutes. The results were recorded as 0, 1, 2, 3, and 4 plus fixation. The usual controls (antigen, antiserum, normal serum) were set up at each titration. In addition, the antigen was periodically checked against both human and rabbit positive sera.

IV. Collection of sera from patients. 10-15 ml of blood was withdrawn from patients receiving the Semple anti-rabies vaccine at the beginning of treatment, and, as nearly as possible, every two to three days thereafter. The blood was allowed to clot, the serum separated by centrifugation, and frozen at –30°C until used.

Experimental. I. Four rabbits, each weighing about 5 lbs, were given 6 intravenous injections of 3 ml of the human brain suspension mixed with normal rabbit serum at 4-day intervals as described. Control sera obtained at the start of the injections showed a + fixation of complement when 0.1 ml of the undiluted serum was used, but no fixation was observed in a 1:5 or higher dilution. The subsequent titers obtained are shown in the accompanying table.[1] It is to be noted that the injections were discontinued on the 24th day. The sera obtained from these animals were used as controls in the subsequent fixation tests with human sera.

II. The sera from the 5 patients receiving the anti-rabies vaccine were then titrated in a similar manner. The results obtained are shown in Fig. 1 and 2.

Discussion of cases. Cases No. 1, 3, and 4. These patients were given the routine 14 daily injections of the Semple anti-rabies vaccine (25% suspension phenol-killed rabbit brain virus) following a dog bite in which the dog was not found for examination. They tolerated the injections well and showed very

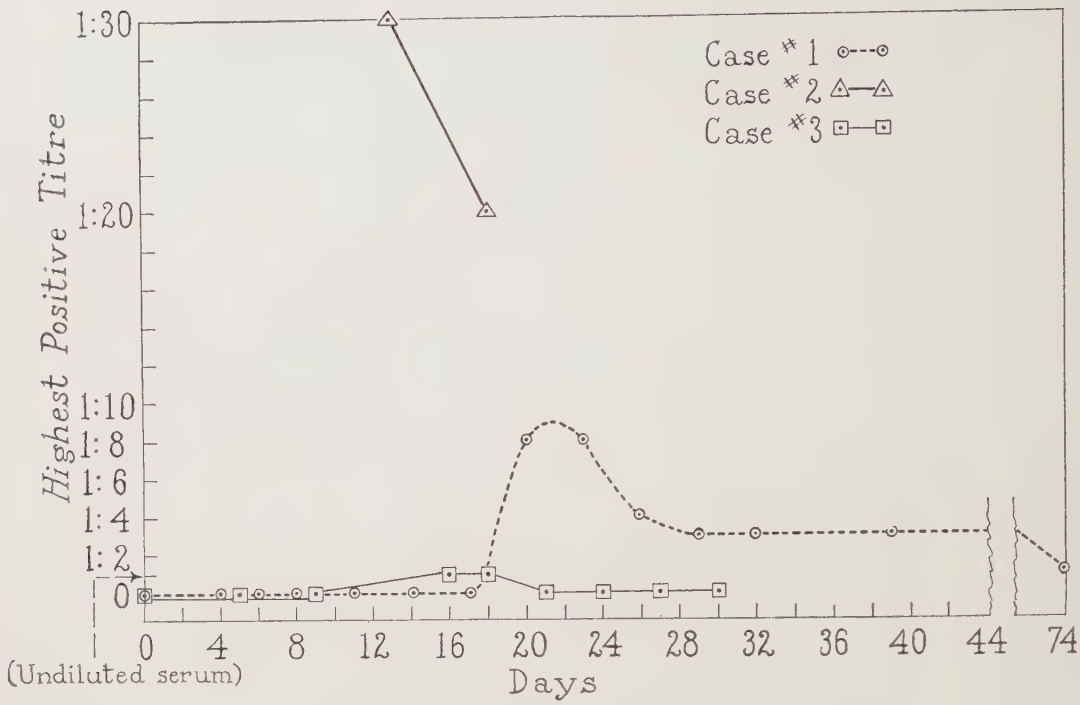

FIG. 1.
Complement fixation titers in cases 1, 2, and 3.

little reaction. Cases 3 and 4 developed some swelling and redness at the site of the injections between the 10th and 14th dose. In each case, the sera gave a negative complement fixation reaction until the 15th to 18th day at which time antibodies to the brain antigen appeared. (See Fig. 1 and 2).

Case No. 2. This patient was a 9-year-old Italian boy who was bitten by a dog on August 17, 1948. The dog was not found and for this reason injections of the antirabies vaccine were started on August 23, 1948. He received a total of 5 injections, each of which produced considerable swelling and redness at the site of injection. Because of the discomfort, the patient and his parents objected strenuously to the treatment, and the patient was discharged against medical advice. He had given no previous history of allergy, and his initial physical examination was entirely normal. On August 29, 1948, 6 days after the first injection with the antirabies vaccine, the patient became lethargic, vomited, developed a fever, anorexia, and pain in the right upper abdominal quadrant. He was readmitted to the hospital where his temperature was found to be 39.2°C, pulse 120, and his appearance lethargic. He appeared "quite ill." Examination revealed a mild inflammation of the nasopharynx, a stiff neck, and a positive Kernig and Brudzinski. The total white count on admission was 9,800 with a slight leukocytosis. The spinal fluid showed 23 cells, of which 18 were described as monocytes and 5 as polymorphonuclear leukocytes. The CSF protein was 70, sugar 100, and chlorides (as NaCl) 734.

It was possible to obtain only 2 samples of his blood for titration, one on September 7th, 9 days following the development of symptoms, and the other 3 days later. Both samples showed a high titer of complement fixation when compared to the other cases studied, the second blood sample showed a falling titer. Apparently, the antibody titer had begun considerably earlier than in the other four cases, and it may well have reached an even higher concentration than that found in the sample of September 7th. The patient's symptoms subsided and he was dis-

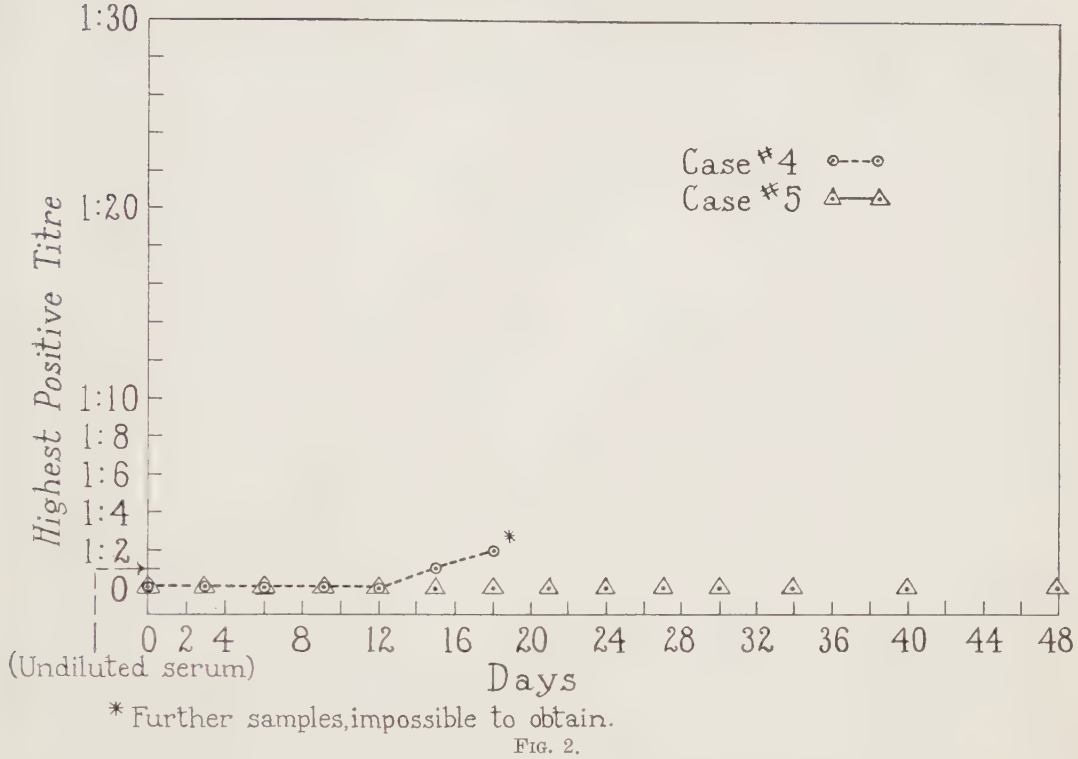

FIG. 2.
Complement fixation titers in cases 4 and 5.

charged entirely well on his 14th hospital day, with the provisional diagnosis of "possible encephalitis following anti-rabies treatment."

Case No. 5. This 34-year-old white female was bitten on the face by a stray dog. She was given the routine 14 daily injections of the vaccine and showed only a slight amount of redness and swelling at the site of injections between the 10th and 14th day of treatment. Blood samples obtained up to the 60th day after the beginning of treatment failed to show fixation of complement. The tests on the sera of this patient were repeated with varying dilutions of the antigen from 1 to 20 up to 1 to 1280, but no fixation occurred. It was then thought that the patient's sera might contain an inhibiting substance. In order to determine this, portions of these same sera (0.3 ml) were mixed with equal portions of the fresh positive serum of Case No. 2. This mixture was inactivated at 55°C for 30 minutes and varying dilutions were then used in fixation reactions with the brain antigen as previously described. Controls of the known positive serum diluted in the same manner with a normal serum, and of the positive serum alone were run at the same time. No inhibition of complement fixation was found to occur with the sera of case No. 5 or with the controls. It was therefore thought that patient No. 5 failed to show antibrain antibodies following treatment with anti-rabies vaccine.

Comment. The present work confirms that of Lewis in showing that antibodies to brain tissue are produced in the rabbit by the intravenous injection of foreign brain substance. It also provides evidence that antibodies to human brain are produced in patients receiving the Semple antirabies vaccine. Case 2 suggests the possibility that a markedly increased antibrain antibody titer may occur in those patients developing an encephalitis following this treatment.

17052

A Plasma Factor Responsible for *in vitro* Lysis of Leucocytes by Tuberculoprotein.*

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From the Medical Clinics, Peter Bent Brigham Hospital, and the Department of Medicine, Harvard Medical School.

Rich and Lewis,[1] studying living tissue cultures of washed leucocytes, showed that tuberculin in proper concentration had a selective toxic effect on cells from tuberculous animals. Since this phenomenon occurred even when allergic cells were suspended in the plasma of a normal animal, it was felt that this manifestation of tuberculin sensitivity was not dependent on circulating plasma antibodies of the sensitized animal but rather was a property inherent in the cells. Subsequent tissue culture studies[2-5] have confirmed this specific cytotoxic effect of tuberculin on leucocytes from tuberculous animals and again the reaction has been attributed to a property of the cells rather than to any serum antibody. Chase[6] and others[7,8] demonstrated that the tuberculin type of hypersensitivity could be transferred passively to normal guinea pigs by injection of the cells of peritoneal exudates, lymph nodes or spleens of guinea pigs sensitized to tuberculin by the injection of heat killed tubercle bacilli, thus confirming some crucial property of cells as the basis of delayed, tuberculin-type hypersensitivity.

More recently, it has been shown[9] that bacterial products of the tubercle bacillus exert a significant lytic effect *in vitro* on leucocytes from tuberculous animals after a one-hour period of incubation. A similar cytotoxic response has been observed on white cells from tuberculous humans.[10] Since these human white cells were suspended in normal serum (or plasma), it was concluded that the cytotoxic action of tuberculin was specific to tuberculous-type white cells and not dependent on any serum component.

It is now apparent that if such tuberculous-type white cells are thoroughly washed and then suspended in normal plasma, no lytic effect by tuberculin can be demonstrated over the one hour period of incubation. But if such washed cells, or even white cells from a normal tuberculin-negative human, are added to the plasma of a tuberculous human in the presence of old tuberculin, white cell lysis occurs during the course of one hour. It is the purpose of this report to demonstrate that a factor in tuberculous plasma is necessary for the lysis of white cells by tuberculin.

Experimental. Using a method previously described,[11] white cells from normal tuberculin-negative humans and from tuberculous patients hospitalized for acute tuberculous infection were concentrated and thoroughly washed with isotonic saline solution. The resulting cell concentrates were then suspended in normal human plasma as well as in tuberculous plasma, the amount of plasma being

* Work done under an U.S.P.H.S. Research Grant.

† Research Fellow, National Institute of Health.

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TABLE I.
In vitro Effect of Tuberculin on Blood Leucocytes in the Presence of Normal and Tuberculous Plasma.

Normal cells + Normal plasma*	0.4†	0.4						
Normal cells + Tuberculous plasma			0.4	0.4				
Tuberculous cells + Normal plasma					0.4	0.4		
Tuberculous cells + Tuberculous plasma							0.4	0.4
Tuberculin antigen	0.1		0.1		0.1		0.1	
Saline		0.1		0.1		0.1		0.1
Total WBC								
5 min.	7,760	7,440	7,810	8,070	11,500	12,150	5,070	4,000
60 min.	7,790	7,450	5,980	8,130	11,466	12,220	3,870	4,050
% decrement	+0.5	+0.3	—23.2	+0.8	—1.0	+0.5	—23.8	+1.2

* Normal cells and normal plasma are obtained from a healthy tuberculin-negative subject.
† Amounts refer to cubic centimeters.

adjusted so that final cell concentrations varied between 4,000 - 15,000 cells per cu mm. To 0.4 cc of such white cell suspensions was added 0.1 cc of tuberculin antigen (prepared as described earlier[11]). As cell system controls, 0.1 cc of isotonic saline was added to a duplicate series of cell suspensions. The test tubes used in these experiments have been coated with organosilicone to diminish nonspecific cytolysis. White blood counts were done with a mechanical pipette filler and calibrated pipettes (15 squares on 2 chambers of the standard hemocytometer slide were counted). Cytolysis was demonstrated by doing total white counts before and after a 60 minute period of incubation at 37°C.

Results. A sample protocol illustrates the results obtained in typical experiments.

1. White cells from normal tuberculin-negative humans as well as from tuberculous patients can be lysed when suspended in the plasma of tuberculous patients in the presence of old tuberculin.

2. Adequately washed tuberculous cells will show no cytolysis by tuberculin when such cells are suspended in normal plasma.

3. Such cytolysis by tuberculin varies between 20-35% of the total cells present under conditions of these experiments.

4. When isotonic saline is substituted for the tuberculin antigen, no cytolysis beyond 2.2% could be demonstrated. This variation in cell count is felt to be the amount of error inherent in the method used.

Discussion. From the foregoing, it seems apparent that tuberculin antigen can exert a cytotoxic effect on washed normal tuberculin-negative human white cells as well as on those of tuberculous patients provided tuberculous plasma (or serum) is present in the system. Results of an earlier report[10] in which tuberculous cells were similarly lysed in normal serum were probably due to the presence of traces of tuberculous plasma on the surfaces of unwashed or insufficiently washed white cells. Since it has been shown[11] that normal tuberculin-negative human white cells have the same capacity to adsorb tuberculin onto their cell surfaces as do tuberculous cells, it would appear that the vital factor in cytolysis of white cells by tuberculin is some component in tuberculous plasma. Further experiments to elucidate the nature and origin of this plasma factor are now in progress.

Summary. Under the conditions of the experiments reported here, the cytolysis of human white blood cells by tuberculin occurs

only in the presence of plasma from tuberculous subjects. White blood cells from healthy tuberculin-negative humans will undergo similar tuberculin cytolysis in the presence of such tuberculous plasma.

17053

The Role of the Spleen in Radiation Injury.*

LEON O. JACOBSON, E. K. MARKS, E. O. GASTON, M. ROBSON, AND R. E. ZIRKLE.
From the Argonne National Laboratory, the Department of Medicine, and the Institute of Radiobiology and Biophysics of the University of Chicago.

Ectopic blood formation in the spleens of mice injected with a dose of 2.0 microcuries per gram of body weight of radiostrontium (Sr^{89}), as shown by Jacobson *et al.*,[1,2] was sufficient to obviate the development of anemia even though the bone marrow was largely destroyed and only gradually reconstituted over a period in excess of 100 days. Splenectomized mice given this dose developed a severe anemia, recovery from which occurred only as the hematopoietic activity of the bone marrow recovered. This communication describes a somewhat different but related technic for studying the significance of the spleen in recovery from or compensation for radiation injury.

Materials and Methods. Four groups of young female mice were prepared as indicated in Table I. Mice in Group I were untreated controls. The mice in Groups II, III, and IV were anesthetized, an incision made in the left upper quadrant of the abdomen, and the spleen brought out through the abdominal incision with the main pedicle intact. Group III and Group IV mice were irradiated with 600 r whole-body X radiation (250 Kv) except that during the irradiation the mobilized spleens of Group IV mice were placed in one-quarter inch thick lead boxes with openings for the pedicle only. The mobilized spleens of Group III mice were placed in thin paraffin boxes which offered no appreciable shielding from the radiation. The mobilized spleens of Group II mice (operated controls) were placed in lead boxes for a period equal to the time that Group III and Group IV spleens were thus contained. The radiation required approximately 12 minutes after which the spleens of groups II, III, and IV mice were returned to the abdominal cavity and the operative incisions sutured.

Results. Hematologic studies were made on all 4 groups. Animals from each group were sacrificed at intervals for histopathologic study.

The mean hemoglobin, erythrocyte, and hematocrit values of Group IV (lead-protected spleens) were not significantly altered when compared to control Groups I and II (Fig.

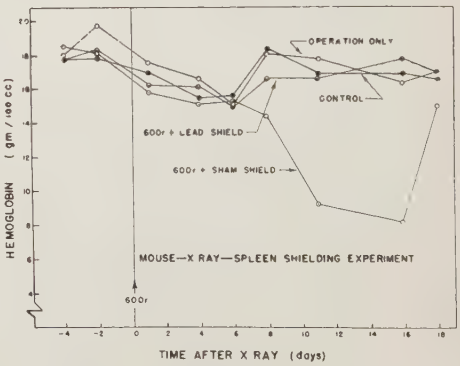

FIG. 1.
The hemoglobin values of control mice and mice exposed to 600 r with and without lead protection of the spleen.

* Aided in part by a grant from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council and a grant from Armour and Company.

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TABLE I.
Preparation and Treatment of Animals.

Group	No. of animals	Preparation of animals	Treatment
I	15	None	None
II	20	Anesthetic (Nembutal) Surgical mobilization of spleen	"
III	20	Anesthetic (Nembutal) Surgical mobilization of spleen	600 r total body X irradiation inclusive of spleen
IV	20	Anesthetic (Nembutal) Surgical mobilization of spleen	600 r total body X irradiation exclusive of spleen

1-3). In Group III (spleens unprotected), however, these values were markedly reduced between the sixth and eighteenth day after irradiation. The mean reticulocyte value of Group III animals (spleens unprotected) was reduced to less than 0.1% by 2 days and remained reduced through nine days (Fig. 4). The mean reticulocyte value of Group IV mice (lead-protected spleens) was not significantly reduced at any time; a definite increase above the normal value occurred between the third and fourteenth day. The mean platelet value of Group III (spleens unprotected) fell gradually to a minimum of 15,000 cu mm on the eighth day and rose to a normal value by the eighteenth day after irradiation, whereas the platelet value of Group IV (lead-protected spleens) reached a minimum of 230,000 cu mm on the ninth day and rose to a normal value by the eleventh day. (Fig. 5) The mean leucocyte value of Group III (spleens unpro-

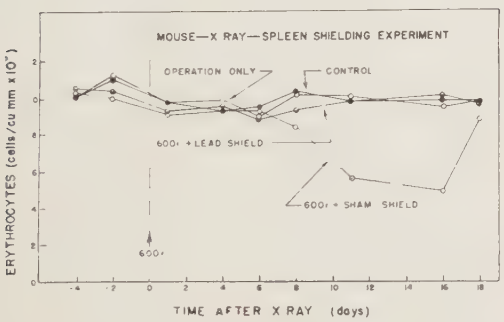

FIG. 2.

The erythrocyte values of control mice and mice exposed to 600 r with and without lead protection of the spleen.

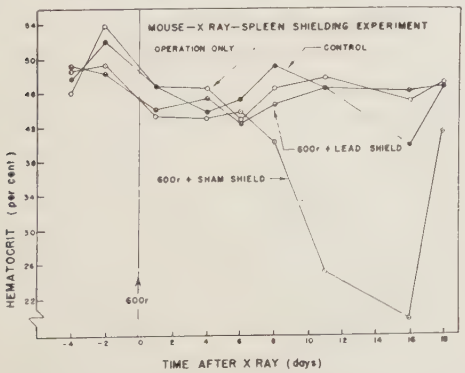

FIG. 3.

The hematocrit values of control mice and mice exposed to 600 r with and without lead protection of the spleen.

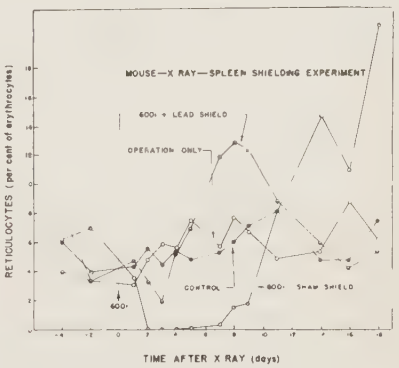

FIG. 4.

The reticulocyte values of control mice and mice exposed to 600 r with and without lead protection of the spleen.

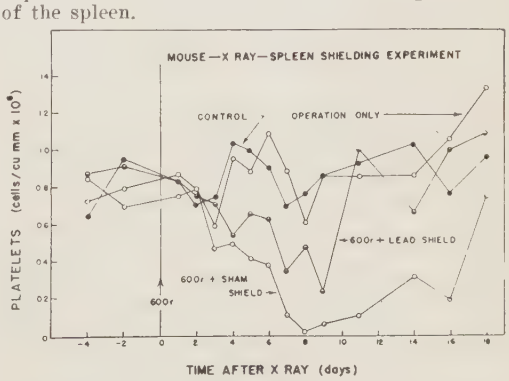

FIG. 5.

The platelet values of control mice and mice exposed to 600 r with and without lead protection of the spleen.

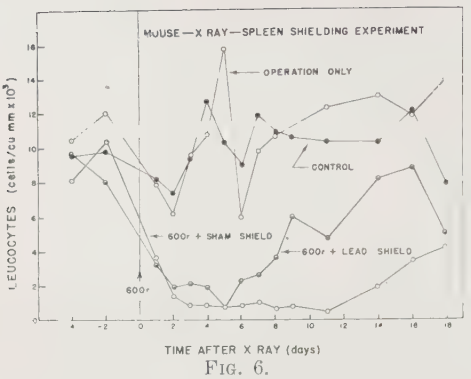

FIG. 6.

The leucocyte values of control mice and mice exposed to 600 r with and without lead protection of the spleen.

tected) was reduced below 1000 per cu mm by the third day and remained below 1000 through the eleventh day after irradiation. The mean leucocyte value of Group IV (lead-protected spleens) fell to a minimum of circa 2000 per cu mm only and rose to a relatively normal value by the ninth day after irradiation (Fig. 6).

The spleens of Group III (spleens unprotected) decreased markedly in size within twenty-four hours and remained thus reduced in size beyond the tenth day after irradiation. The spleens of Group IV (lead-protected spleens) increased in size reaching in some instances approximately twice the size of controls by the third day.

The histologic studies revealed that hematopoietic tissue in Group III animals that received 600 r inclusive of the spleen was largely destroyed with significant regeneration only beginning after the sixth day. A comparable degree of destruction of hematopoietic tissue occurred in Group IV animals except in the lead-protected spleens where a marked increase in erythrocytopoiesis, megakaryocytopoiesis, and granulocytopoiesis was already apparent by 18 hours after exposure. This ectopic blood formation in the spleen increased rapidly in extent. Lymphatic tissue in these lead-protected spleens decreased however, as the erythro-, granulo-, and megakaryocytopoiesis increased. By forty-eight hours after irradiation the amount of lymphatic tissue remaining in the lead-protected spleens was approximately 75% less than controls and consisted largely of medium and small lymphocytes about the arterioles in the white pulp.

Summary and conclusions. These hematologic and histologic data indicate that:

1) Severe anemia, leucopenia, and thrombocytopenia develop in mice after a single dose of 600 r whole-body X radiation.

2) Ectopic erythrocytopoiesis, in the lead-protected spleens of mice given 600 r whole-body X radiation (exclusive of spleens) compensates with such rapidity and so extensively for the destruction and interruption of this activity in the marrow spaces that no anemia of significance becomes apparent. Ectopic granulocytopoiesis and megakaryocytopoiesis in the lead-protected spleens compensates significantly but at a slower pace and less completely for the bone marrow destruction.

3) A marked and sustained decrease in the amount of lymphatic tissue is produced in the lead-protected spleens of animals given 600 r whole-body X radiation. This decrease in lymphatic tissue may perhaps be a result of (a) unsuccessful competition of the lymphatic tissue with the ectopic hematopoiesis for nutritional requirements, (b) actual indirect effect of radiation and (c) a differential humoral suppression from some unknown site.

The rapidity with which erythrocytopoiesis transfers from the X-ray damaged bone marrow to the lead-protected spleen in the absence of anemia suggests that the mechanism of stimulation of erythrocytopoiesis under the conditions of this experiment may involve some factor or factors other than, or in addition to, the accepted hemoglobin-oxygen relationship.

This technic permits more or less exclusive protection of the spleen or the appendix or other visceral tissues from irradiation while applying various dosages to the remainder of the body. It provides a method of studying potential sites and mechanism of the production of ectopic blood formation, possible secondary effects of radiation as well as offering possibilities for determining the potential role of such sites in immune reactions, in preventing or alleviating radiation-induced hemorrhagic phenomena and in the study of survival or recovery from radiation injury.

17054

Proteolytic Activity of Hemophilic Plasma.

DAN A. RICHERT. (Introduced by W. W. Westerfeld.)

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Tagnon, Davidson, and Taylor[1] reported that the proteolytic activity of hemophilic plasma was lower than normal, but subsequently this group of investigators found no such difference.[2] In both studies the proteolytic enzyme was activated by treatment with chloroform; proteolytic activity was then measured by the rate of destruction of fibrinogen and fibrin[1] or by the rate of digestion of casein.[2]

Human blood plasma contains the inactive proteolytic enzyme precursor, plasminogen,*[3] which can be activated partially at least by chloroform treatment; activation with streptokinase appears to be much more effective. Plasma also contains an antiproteolytic factor[7-9] that must be removed or destroyed in order to obtain a true measure of the enzyme activity. In the procedure herein described the plasminogen has been separated from the antiproteolytic material by an alcohol fractionation similar to that devised by Cohn *et al.*[10,11] for the separation of plasma proteins. The enzyme was then activated with streptokinase,[12] and the plasmin activity was estimated from a measurement of fibrinogenolysis time.[1,13] A comparison of hemophilic and normal plasmas showed little if any difference in plasminogen content.

Experimental. Reagents. 1. 50% alcohol. 500 ml of 95% ethanol were diluted with distilled water to 950 ml.

2. Acetate buffer, pH 4. 12 ml of 1 M sodium acetate and 72 ml of 1 M acetic acid were mixed and diluted to 1 liter with distilled water.

3. Phosphate buffer, pH 10.1, with sodium citrate. 13.4 g of $Na_2HPO_4 \cdot 7H_2O$ and 29.4 g of $Na_3C_6H_5O_7 \cdot 2H_2O$ were dissolved in approximately 500 ml distilled water. Normal sodium hydroxide was added to adjust the pH to 10.1, and the solution was then diluted to 1 liter.

4. Phosphate buffer, pH 7.2, with sodium citrate and sodium chloride. 4.14 g of $NaH_2PO_4 \cdot H_2O$, 8.82 g of $Na_3C_6H_5O_7 \cdot 2H_2O$ and 8.91 g of NaCl were dissolved in approximately 500 ml distilled water. The pH of the solution was adjusted to 7.2 with normal sodium hydroxide, and the solution was then diluted to 1 liter.

5. Streptokinase. A crude preparation of this enzyme was prepared from a β-hemolytic streptococcus medium by the alcohol precipitation method of Garner and Tillett.[14] The precipitate was dried from the frozen state

1 Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.*, 1943, **22**, 127.

2 Lewis, J. H., Davidson, C. S., Minot, G. R., Soulier, J. P., Tagnon, H. J., and Taylor, F. H. L., *J. Clin. Invest.*, 1946, **25**, 870.

* Several proposals for the nomenclature of the active enzyme and its precursor have been made. They are a) plasmin and plasminogen;[3] b) fibrinolysin and profibrinolysin;[4] c) serum tryptase and tryptogen;[5] d) serum protease and lytic factor[6] respectively.

3 Christensen, L. R., and MacLeod, C. M., *J. Gen. Physiol.*, 1945, **28**, 559.

4 Loomis, E. C., George, C., Jr., and Ryder, A., *Arch. Biochem.*, 1947, **12**, 1.

5 Ferguson, J. H., *Science*, 1947, **105**, 488.

6 Kaplan, M. H., *J. Clin. Invest.*, 1946, **25**, 331.

7 Christensen, L. R., *J. Gen. Physiol.*, 1946, **30**, 149.

8 Grob, D., *J. Gen. Physiology*, 1943, **26**, 405.

9 Kaplan, M. H., *J. Clin. Invest.*, 1946, **25**, 337.

10 Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, 1946, **68**, 459.

11 Edsall, J. T., *Advances in Protein Chemistry*, 1947, **3**, 383.

12 Christensen, L. R., *J. Gen. Physiol.*, 1945, **28**, 363.

13 Ferguson, J. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 243.

14 Garner, R. L., and Tillett, W. S., *J. Exp. Med.*, 1934, **60**, 239.

TABLE I.
Fibrinogenolytic Activity of the Plasmin Fraction of Normal and Hemolytic Plasmas Activated with Streptokinase.

	Subject	Amt of streptokinase added to 5 ml of solution (mg)	Fibrinogenolysis time: % conc. of plasma 100 Min.	50 Min.	12.5 Min.
Normal Plasma Preparations	1	25	4		21
	2	25	5		15
	3	25	4		8
	4	25	8		35
	5	25	4		18
Hemophilic Plasma Preparations	1	25	7		18
	2	25	6		19
	3	25	No act.		41
		75	—	7	—
		200	6		—
Beef fibrinogen		25	Good clot formation after 21 hr		
		200	Good clot formation after 21 hr		

and resuspended in the phosphate buffer just before use.

6. Beef fibrinogen was prepared according to Ware, Guest, and Seegers.[15] A 0.4% solution was made up in the phosphate buffer, pH 7.2. This fibrinogen was free of any plasminogen that could be activated by streptokinase.

7. Thrombin. Sufficient dried Harvard Fraction III-2† was dissolved in 0.85% sodium chloride so that 0.2 ml clotted 0.2 ml of a 0.4% fibrinogen solution in approximately 1 minute.

Plasma Fractionation Procedure. Citrated blood (12 ml whole blood and 1 ml 4% sodium citrate) was centrifuged; 5 ml of the plasma were measured into a 50 ml centrifuge tube and cooled to 1°C in an ice bath. 2.5 ml of cold acetate buffer were added, giving a pH of approximately 6.8. 12.5 ml of the cold ethanol solution were then added slowly from a pipette with constant stirring to give a precipitate containing the fibrinogen, plasminogen, antiproteolytic material and other proteins. After standing in an ice bath for 30 minutes the precipitate was removed by centrifuging in the cold (3-5°C), and the supernatant solution was discarded.

The precipitate was then suspended in 0.3 ml of phosphate buffer, pH 10.1, and the suspension was dissolved by the addition of 5 ml of cold water. To the solution were added 10 ml of cold water and 15 ml of cold ethanol solution. The pH of this medium was about 7.6. It was cooled in an ice bath for 30 minutes, centrifuged in the cold, and the supernatant solution (containing the antiproteolytic material) was discarded. The precipitate was redissolved in phosphate buffer, pH 7.2, and the final volume was adjusted with buffer to 5 ml. The buffer was prewarmed to 38°C, and was added just before the sample was treated with streptokinase.

This solution, containing the original plasma concentration of plasminogen and fibrinogen, was tested at both 100% and 12.5% of the original plasma concentration. In testing the undiluted solution (100%) the fibrinogen of the original plasma served as the substrate. In testing at 12.5% concentration, 1 ml of the solution was diluted with 7 ml of the beef fibrinogen substrate. By including cit-

[15] Ware, A. G., Guest, M. M., and Seegers, W. H., *Arch. Biochem.*, 1947, **13**, 231.

† The fraction III-2 was obtained through the courtesy of Dr. John T. Edsall. It was prepared under a contract between the Office of Scientific Research and Development and Harvard University from human blood collected by the American Red Cross.

rate in the buffer reagents the fibrinogen did not clot until thrombin was added.

Activation and Test for Proteolytic Activity. 5 ml of the solution to be tested were placed in a constant temperature bath at 38°C for 5 minutes; 0.2 ml of the streptokinase suspension was then added. Aliquots of 0.2 ml were removed at one-minute intervals following the streptokinase addition, and these were immediately tested for clotting power by the addition of 0.2 ml of thrombin solution. The time at which thrombin no longer clotted the test solution was recorded as the fibrinogenolysis time. This was the time required for the streptokinase to activate the enzyme and for the latter to digest the fibrinogen substrate to the point where it no longer coagulated.

Results. Table I shows the fibrinogenolytic activities of 3 hemophilic plasmas and of 5 normal human plasmas.

The fraction obtained from hemophilic patient No. 3 showed no activity when the 100% solution was treated with 25 mg of streptokinase. However, good proteolytic activity was demonstrated in this plasma when the relative proportion of streptokinase to plasma was greatly increased. This suggests that the fraction from this plasma contained a high concentration of antistreptokinase[16] which could be overcome by treatment with large amounts of streptokinase.

Discussion. By the precipitation of plasminogen from plasma under the conditions described, its activation by streptokinase and its proteolytic activity could de demonstrated without interference from the antiproteolytic factor. When whole plasma was treated with streptokinase in the same proportion as was used with fractionated plasma, this rapid digestion of fibrinogen was not observed. Neither could a solution of the first alcohol precipitate of plasma be rapidly activated by streptokinase.

Christensen and MacLeod[3] and Kaplan[17] have shown that the chloroform activated and the streptokinase activated proteolytic enzymes are identical. Since the streptokinase activated proteolytic activity of hemophilic plasma in this experiment was found to be comparable to that of normal human plasma, the results confirm the conclusions of Lewis, *et al.*,[2] that the proteolytic activity of hemophilic plasmas is not impaired.

Summary. 1. A method is described by which the proteolytic activity of human normal and hemophilic plasmas may be compared.

2. The proteolytic activity of 3 hemophilic plasmas was found to be comparable to that of normal plasmas.

Gratitude is expressed to Dr. Tyree C. Wyatt and Dr. John Houpis of the Pediatrics Departments, Syracuse University College of Medicine and Syracuse Memorial Hospital for their cooperation in securing the bloods from the hemophilic patients.

16 Kaplan, M. H., in collaboration with the Commission on Acute Respiratory Diseases, *J. Clin. Invest.*, 1946, **25**, 347.

17 Kaplan, M. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 1940.

17055

Effect of Boric Acid on Biological Activity of Alloxan.

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In the course of studies on the effect of dietary factors in alloxan diabetes it was found[1] that when rats on a tocopherol deficient diet are injected with alloxan they exhibit marked hemoglobinemia and hemoglobinuria. This reaction has not been observed in animals receiving tocopherol. Because he thought it might be of interest to use in connection with this problem, Professor Richard Kuhn (Heidelberg) kindly sent to us in advance of publication the results of a study by Kuhn and Quadbeck[2] on the effect of simultaneous administration of alloxan and boric acid. These authors found that when boric acid was administered with alloxan, blood sugar values were lower and survival time longer than when alloxan was given alone. When a second injection of alloxan and boric acid was given a week later, however, severe diabetes was produced. Boric acid is known to increase the activity of alloxan in its lactin form[3,4] and they attribute the protective effect of boric acid to increased reactivity of alloxan in other active centers so that less reaches the pancreas; failure of protection on reinjection may indicate saturation or inactivation of these centers. We have carried out experiments similar to those of Kuhn and Quadbeck on animals receiving our special rations, and have studied hemolysis as well as diabetes.

Experimental. The general procedure in this study was the same as that previously reported.[1] Two types of diet were used: a high-lard diet (casein 20%, sugar 36%, lard 38%, cod liver oil 2%, salt mixture 4%) which in the previous experiment had caused marked hemolysis and high mortality, and a low-fat diet (casein 20%, sugar 76%, salt mixture 4%, supplemented with 3 drops of corn oil per day and 3 drops of percomorph oil per week) where hemolysis and mortality were low and there was consequently better opportunity of observing the development of diabetes. All animals received a daily supplement of crystalline B-vitamins and the tocopherol-treated groups received 3 mg of mixed tocopherols* daily. Female rats of the Sprague-Dawley strain weighing 100 to 145 g were used. They were kept on the experimental diet for a month before injection of alloxan.

Alloxan and boric acid (10-16 mg/cc) were injected intraperitoneally at a level of 160 mg/kg each. When both were given they were injected separately but almost simultaneously. Blood sugars were taken the second day after injection and determined by the method of Somogyi[5] as modified by Nelson.[6,†] In some cases blood NPN was also determined. Hemolysis was estimated by hematocrit determinations 15 and 30 minutes after injection and by the amount of hemoglobin observed in the urine which was collected on filter paper.

The study previously reported[1] had furnished a large number of control animals so only a few injections of alloxan alone were made in the present series. These special control animals were used for simultaneous

[1] György, P., and Rose, C. S., *Science*, 1948, **108**, 716.

[2] Kuhn, R., and Quadbeck, G., unpublished.

[3] Kuhn, R., and Weygand, F., *Ber. d. deutsch. chem. Gesellsch.*, 1935, **68**, 1282.

[4] Kuhn, R., Reinemund, K., Weygand, F., and Ströbele, R., *Ber. d. deutsch. chem. Gesellsch.*, 1935, **68**, 1765.

* Kindly furnished by Distillation Products, Inc., Rochester, N. Y.

[5] Somogyi, M., *J. Biol. Chem.*, 1945, **160**, 61.

[6] Nelson, N., *J. Biol. Chem.*, 1944, **153**, 375.

† The blood sugar determinations were made in the George S. Cox Institute through the courtesy of Dr. F. D. W. Lukens.

TABLE I.
Effect of Boric Acid Injected with Alloxan on Survival and Diabetes.

Diet	No. of rats	Treatment*	Survival (%) 2 days	Survival (%) 7 days	Blood sugar, 48 hr (mg %)
High lard	10	A	50	10	405 (350-478)
" "	8	A + B	100	88	151 (114-213)
High lard—tocopherol	10	A	60	10	461 (376-643)
" " "	4	A + B	100	75	142 (111-135)
Low fat	6	A	100	83	467 (230-898)
" "	6	A + B	100	100	123 (111-136)
Low fat—tocopherol	5	A	100	80	612 (479-873)
" " "	5	A + B	100	100	134 (94-200)

* A = Alloxan, 160 mg/kg; B = Boric acid, 160 mg/kg.

comparison of hemolysis caused by alloxan with and without boric acid.

Results. In Table I are given the blood sugar and survival data for rats receiving alloxan and alloxan with boric acid. In every group those animals which received alloxan with boric acid had nearly all normal blood sugars, while all receiving alloxan alone were diabetic. When alloxan was injected survival was much better in the groups receiving the low-fat ration than in those which had the high-fat diet although the diabetes was equally severe in both cases. When boric acid was injected with alloxan there was definite improvement in survival in the groups on the high-lard diet. There was no mortality during the first 2 days following injection, and only 2 animals of the 12 died within 7 days, while with alloxan alone 18 out of 20 had died during this period. All animals on the low-fat diet which had been injected with alloxan and boric acid survived the 7 day period, but with alloxan alone, only 2 of 11 had died. In contrast to the animals which received alloxan alone blood NPN was never elevated in rats treated with the combination of alloxan and boric acid.

We found much less serious effects following a second injection than did Kuhn and Quadbeck. Of the whole series of animals, only 3 died after the second injection, all within the first 2 days. Two had been on the high-lard diet, one of the low-fat diet. None of the survivors showed elevated blood sugar levels and urine sugars determined a week or so after injection were almost all negative, with no more than a trace of sugar found in any sample. In Table II are given values found on animals which received 2 to 4 injections of alloxan with boric acid. In no case was there an elevated sugar, but when alloxan was given a diabetic sugar level was found in those animals which survived for 2 days following the injection.

The primary purpose of Table II is comparison of hemolysis following injection of alloxan with and without boric acid. Hemolysis is never observed in animals receiving tocopherol and is relatively mild in animals on a low-fat diet. Therefore, all of the animals considered here were on the high-lard ration without tocopherol. The first animals observed showed only mild hemoglobinuria, an estimated trace to +, as compared with an almost uniform ++ to ++++ in animals previously observed which had been injected with alloxan alone. However, since the observations were only semi-quantitative and there might be some difference in interpretation and, of greater importance, since we had found that tocopherol intake in the pre-experimental period might have some effect, simultaneous injections of alloxan and alloxan with boric acid were made. Five such pairs were studied, 2 on a first injection, 3 others by injecting one of a pair of animals which had both previously been injected with alloxan plus boric acid with alloxan alone, while the other again received alloxan with boric acid. In every case hemolysis as estimated from hemoglobinemia and hemoglobinuria was ++ or ++++ when alloxan was given

TABLE II. Blood Sugar and Hemolysis in Rats on a High Lard Diet Injected with Alloxan and Alloxan with Boric Acid.

Rat	1st injection			2nd injection			3rd injection			4th injection		
	Date treatment*	Hemolysis	Blood sugar 48 hr (mg %)	Date treatment	Hemolysis	Blood sugar 48 hr (mg %)	Date treatment	Hemolysis	Blood sugar 48 hr (mg %)	Date treatment	Hemolysis	Blood sugar 48 hr (mg %)
1	1/25	—	147	2/2	+	145	2/6	++++	†			
	A + B			A + B			A					
2	1/25	+	213									Dead 1/28
	A + B											
3	1/25	+	155	2/2	±	141	2/6	—	159	2/12	+++	380
	A + B			A + B			A + B			A		
4	1/26	+++	*									
	A											
5	1/26	+	194	2/3	±	155	2/6	++++	†			
	A + B			A + B			A					
6	1/27	+++	732									Dead 1/31
	A											
7	1/27	—	125	2/3	—	134	2/6	—	139	2/12	+	119
	A + B			A + B			A + B			A + B		

* A = Alloxan, 160 mg/kg; B = Boric acid, 160 mg/kg. † Dead within 48 hours following injection.

alone, never more than + when boric acid was given simultaneously.

Discussion. Production of diabetes is not the only known biological effect of alloxan. Before this property was known it had been studied as a capillary poison and spasmodic. Labes and Freisburger[7] made a study of the chemical properties of alloxan from this point of view. Kidney damage has been frequently observed in connection with alloxan diabetes. Houssay and Martinez[8] studied the toxicity of alloxan with reference to diet and among other findings noted that low-fat rations protected against early mortality following injection. Data in the present paper confirm this finding but indicate no difference in severity of diabetes with low or high fat in the diet (Table I). The earlier report of the authors[1] indicated another factor in alloxan effect again related to diet: marked hemolysis following alloxan injection in animals deficient in tocopherol. There was no difference in the diabetes produced in the presence or absence of tocopherol (Table I) and little difference in survival except for those tocopherol-deficient animals which died of anemia within a day or so after injection.

When boric acid was given with alloxan there was decreased effect of alloxan in the 3 factors studied: diabetes, mortality, and hemolysis. Kuhn and Quadbeck's suggestion that the increased activity of alloxan in the presence of boric acid results in its dissipation before it reaches the pancreas seems unlikely in view of the decreased effect on mortality and hemolysis. Alloxan disappears from the blood stream very rapidly[9] and the production of diabetes depends on its concentration during the first few minutes following injection. The 160 mg/kg dose of alloxan is not far above the minimum effective dose for intraperitoneal injection. Another possible mode of action of boric acid would be formation of an alloxan-boric acid complex

[7] Labes, R., and Freisburger, H., *Arch. f. exp. Path. u. Pharmakol.*, 1930, **156**, 226.

[8] Houssay, B. A., and Martinez, C., *Science*, 1947, **105**, 548.

[9] Leech, R. S., and Bailey, C. C., *J. Biol. Chem.*, 1945, **157**, 525.

which would reduce the effective concentration of alloxan at any time.

Our results confirmed completely those of Kuhn and Quadbeck with regard to the effect of a first injection of alloxan with boric acid. We are unable to account satisfactorily for the discrepancy between our results and theirs on second injection. They found the second injection much more toxic than the first while we observed this in only a few cases. No effect of alloxan could be seen from most of our blood sugar values, but the mild hemolysis showed that it was not always completely inactivated. A somewhat more serious damage to the pancreas might have occurred in the animals of Kuhn and Quadbeck, not sufficient to cause frank diabetes but leaving them more susceptible to injury by the second dose. This is more probable since their dose of alloxan varied from 150 to 250 mg/kg while ours was always 160 mg/kg.

Summary. When rats on special rations were injected simultaneously with alloxan and boric acid, the deleterious effects of the alloxan were reduced. Only a few animals showed mild diabetes while alloxan alone produced severe diabetes in all cases. Survival for 7 days in animals receiving a high-lard diet was raised from 10% to 80%. Intravascular hemolysis in tocopherol-deficient animals on the high-lard diet, always severe with alloxan alone, was absent or mild when the boric acid was given.

17056

Transfusion of Leukocytes and Products of Disintegrated Leukocytes.*

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It is a common clinical observation that no demonstrable rise in leukocyte count can be obtained despite repeated transfusions of whole blood, even when given to individuals with marked leukopenia. It has been thought that this failure to successfully increase the leukocyte count by transfusion might be due either to the short life span of leukocytes, to their rapid disappearance from stored blood or possibly to type incompatibilities. This investigation was undertaken to determine whether transfused leukocytes would remain in the blood stream, and if not, to study the mechanism of their removal.

Methods. White blood cells were obtained from the rabbit's peritoneal cavity by a modification of the method of Mudd and coworkers.[1] 300 to 500 cc of physiologic saline were injected into the peritoneal cavity in the evening and a similar amount of saline the next morning about 15 hours later. Overdistension of the rabbit's peritoneum is poorly tolerated and may result in death of the animal. Four hours after the second injection of saline, the fluid was removed from the peritoneal cavity through a 16 gauge needle, using 0.5 cc (5 mg) of heparin as an anticoagulant. The fluid obtained was centrifuged at a low speed for 5 minutes and the sediment resuspended in 10 to 20 cc of Tyrode's solution so that the leukocyte count was in the neighborhood of 50,000 cells per cmm. This cell suspension was then administered intravenously to the same rabbit (autotransfusion) or into other rabbits (heterotransfusion). The cell free peritoneal fluid was also administered intravenously. The cells obtained by this method exhibited apparently normal ameboid movement, were actively phagocytic for *staphylococcus albus,* and took up vital stains. About 90 per cent

* This work was supported in part by a grant from the National Vitamin Foundation under the direction of Dr. A. D. Welch.

1 Mudd, S., Lucké, B., McCutcheon, M., and Strumia, M., *J. Exp. Med.*, 1929, **49**, 779.

TABLE I.
Autotransfusion: Transfusion of White Cells from Rabbit's Peritoneal Cavity into Ear Vein of Same Rabbit.

Rabbit No.	Control WBC ×100	Max. leukopenia ×100	Time onset leukopenia (min.)	Duration leukopenia (hr)	% leukopenia	Max leukocytosis ×100	Time onset leukocytosis (hr)	% leukocytosis
12	45	15	1	2	67	123	6	173
20	191	51	5	1	73	321	3	68
RP 1	59	12	1	4	65	(52)*	—	—
21	223	47	10	1	88	530	3	133
25	128	31	1	5	76	(100)*	—	—
26	104	44	5	5	58	(71)*	—	—
569	51	—	—	—	—	192	1	276
29	211	26	1	5	89	(82)*	—	—
31	70	18	1	½	73	148	4	116
33	134	39	1	3	70	(198)*	—	—
41	51	16	1	1	69	(50)*	—	—
43	32	7	1	1	78	81	3	149
46	30	19	1	1	38	85	6	179
48	58	32	10	1	45	(87)*	—	—
53	36	—	—	—	—	135	7	305
55	43	7	1	½	84	79	6	84
Avg	91.75	26.32	2.8	2.2	69.3	188.33†	4.3†	164.8†

* The figures in parenthesis are those which were not considered to represent a significant leukocytosis.
† Includes only those with a significant leukocytosis.

TABLE II.
Heterotransfusion: Transfusion of White Cells from Rabbit's Peritoneal Cavity into Ear Vein of Another Rabbit.

Rabbit No.	Control WBC ×100	Max. leukopenia ×100	Time onset leukopenia (min.)	Duration leukopenia (hr)	% leukopenia	Max leukocytosis ×100	Time onset leukocytosis (hr)	% leukocytosis
30	166	55	5	½	68	326	4	96
32	190	30	5	6	79	(148)*	—	—
34	67	37	5	2	43	151	3	122
40	235	54	1	2	79	748	3	218
44	268	65	1	4	75	(282)*	—	—
45	95	29	1	3	69	263	5	175
47	74	18	5	2	75	129	7	73
49	346	28	1	1	91	1262	3	235
54	158	20	1	2	87	(184)*	—	—
56	80	21	1	2	73	(88)*	—	—
57	108	28	1	4	74	(102)*	—	—
RP 9	118	51	1	2	57	194	3	64
RP 11	108	34	1	1	68	299	2	177
RP 13	119	44	1	1	62	(152)*	—	—
RP 15	81	7	1	2	79	(111)*	—	—
RP 16	93	19	1	4	74	(128)*	—	—
RP 17	103	32	5	2	69	248	3	208
Avg	144.17	33.70	2.2	2.2	72	403.50†	3.7†	152†

* The figures in parentheses are those which were not considered to represent a significant leukocytosis.
† Includes only those with a significant leukocytosis.

of the cells were mature neutrophils.

Disintegrated leukocytes were obtained by subjecting the resuspended leukocytes to supersonic vibration for 1 hour at 0°C. The material obtained was centrifuged and the supernatant fluid, free of particulate matter, was administered intravenously to rabbits.

Results. Transfusion of leukocytes into the ear vein of the same rabbit from which they had been obtained resulted in a sudden profound leukopenia in 14 of 16 rabbits (Table I). A transient unsustained drop in

TABLE III.
Transfusion of Disintegrated White Cells.

Rabbit No.	Control WBC ×100	Max. leukopenia ×100	Time onset leukopenia (min.)	Duration leukopenia (hr)	% leukopenia	Max. leukocytosis ×100	Time onset leukocytosis (hr)	% leukocytosis
6	168	43	1	3	74	333	4	98
7	225	64	1	½	71	448	1	98
12	172	45	1	5	79	(142)*	—	—
14A	152	24	1	6	81	(176)*	—	—
14B	175	30	1	6	88	(174)*	—	—
15A	34	11	1	2	69	143	3	379
15B	183	84	1	½	97	476	1	105
16	78	16	1	5	79	(74)*	—	—
17	267	27	1	1	90	389	2	49
21	173	33	1	½	80	286	1	65
Avg	163.05	37.70	1	2.95	80.8	345.9†	2†	132†

* The figures in parentheses are those which were not considered to represent a significant leukocytosis.

† Includes only those with a significant leukocytosis.

TABLE IV.
Summary of Data.

Type of transfusion	Avg control WBC	% developing leukopenia	Avg leukopenia	Avg % drop in WBC	Avg leukocytosis	% developing leukocytosis	Avg % increase in WBC
Autotransfusion	9,175	88	2,632	69.3	18,833	56	164
Heterotransfusion	14,417	100	3,370	71	40,350	53	152
Disintegrated WBC's	16,305	100	3,770	80.8	34,590	60	132

the leukocyte count occurred in the other 2 animals. An initial elevation of the leukocyte count did not occur in any animal. The average time of onset of the leukopenia was 2.8 minutes, the average duration 2.2 hours and the average drop in leukocyte count 69.3% of the control count. In 9 of the 16 rabbits (56%) a subsequent leukocytosis occurred. In these rabbits the average rise in leukocytes was 164.8% of the original leukocyte count and the average time of onset was 4.3 hours after the transfusion was given.

When the cell suspension was transfused into the ear vein of another rabbit, a marked leukopenia developed rapidly in all instances (Table II). The average drop in white count was 72% of the control count, the average time of onset 2.2 minutes, and the average duration 2.2 hours. In 9 of 17 rabbits a subsequent significant rise in leukocyte count occurred. The average maximum rise in leukocyte count was 152% of the initial value and the average time of onset was 3.7 hours.

Intravenous administration of the supernatant fluid obtained by centrifugation of disintegrated leukocytes also produced a leukopenia in all instances (Table III). The average drop in white count was 80.8% of the control count, the average time of onset was 1 minute, and the average duration was 2.95 hours. A subsequent leukocytosis occurred in 6 of 10 rabbits. The average maximum rise in white count was 132% of the control count and the average time of onset of the rise in white count was 2.0 hours. The results of autotransfusion, heterotransfusion and transfusion of disintegrated white cells are summarized in Table IV.

The drop in white count was accompanied by a decrease of approximately 20% in neutrophils and a corresponding increase in the percentage of lymphocytes in the differential count. During the phase of leukocytosis there was a marked increase in the number of neutrophils (to 90% or more) with a shift to the left.

Administration of normal saline, Tyrode's solution, whole blood, heparin, killed typhoid bacilli and a solution of desoxyribosenucleic acid intravenously did not proluce any sig-

nificant change in the leukocyte count. 10 to 15.0 cc of a 1.1% solution of sodium citrate always produced a marked leukopenia but was never followed by leukocytosis.

Mixture of whole blood and white blood cell suspension *in vitro* resulted in the calculated rise in the total white count with no significant change in the count over a period of several hours. It was not possible to demonstrate any differences in white blood cell types by cross-matching with the sera from various recipient rabbits.

Transfusions with intact leukocytes as well as with products of leukocytic disintegration frequently resulted in sudden death, preceded by nystagmus and convulsions in all cases. Rabbits used for repeated transfusions lost weight, appeared chronically ill, and frequently died. The cellular debris from disintegrated white cells appeared to be the most toxic of the substances used. When cell suspensions contained a high percentage of cells showing toxic granulation and disintegrating cells as determined by examination of smears, toxic reactions appeared to be more common than if such were not the case. The thromboplastic activity of leukocytes disintegrated by supersonic vibration was determined. The greatest thromboplastic activity was found in the centrifuged sediment. 0.1 cc of the material was equivalent to 0.940 mg of Maltine thromboplastin; in contrast, 0.3 cc of the supernatant material from disintegrated leukocytes contained less than 0.1 mg. In spite of the high content of thromboplastin, intravenous clotting was not observed in any animal.

Because of the histamine content of leukocytes[2] the effect of intravenous histamine phosphate on the white count was investigated. 0.2 to 0.6 mg of histamine phosphate produced a mild delayed leukopenia in 3 of 10 rabbits. Neither the time of onset, the degree of leukopenia nor the duration of the leukopenia corresponded to that produced with the transfusion of intact leukocytes or the products of their disintegration. One mg of histamine intravenously resulted in sudden death with nystagmus and convulsions. The intravenous administration of an anti-histaminic (pyranisamine maleate) did not prevent the characteristic leukopenia which developed with the transfusion of a white cell suspension in 2 rabbits.

Discussion. It is evident that there is a potent substance (or substances) present in leukocytes obtained from the rabbit's peritoneal cavity which is capable of affecting the level of the leukocyte count of circulating blood. Not only do the transfused cells disappear from the circulation but white cells circulating prior to the transfusion disappear as well. This, together with the results obtained with particle-free solutions of lysed cells would indicate that the effect is more than a mere filtering of foreign bodies by the reticuloendothelial system.

The fate of the transfused cells has been studied in preliminary experiments using leukocytes labeled with radioactive phosphorus. With this method the greatest concentration of radioactivity is found in the lungs. This finding is confirmed by histologic studies of lungs removed at the time of maximum leukopenia. The lung capillaries were found to contain large numbers of leukocytes without edema or fibrin formation. The liver and spleen also contained increased numbers of leukocytes but this finding was not as marked as in the lungs.

Menkin[3,4] has described a leukopenic and leukocytic factor, as well as a toxic substance called necrosin, obtainable from inflammatory exudates. It is possible that these substances obtained from exudates are derived entirely from disintegrating leukocytes. It is noteworthy that effects obtained by administration of leukocytes produced with physiologic salt solution are similar to those obtained by exudates produced with irritants.

It cannot be stated whether leukocytosis is a response to a specific agent contained in the transfused leukocytes or their products, whether it is a response on the part of the bone marrow initiated by the leukopenia or whether it is a spontaneous variation. The fact that leukocytosis does not develop in all

[2] Code, C. F., *J. Physiol.*, 1937, **90**, 485.

[3] Menkin, V., *Arch. Path.*, 1946, **42**, 154.

[4] Menkin, V., *Science*, 1947, **105**, 538.

animals in which leukopenia has been produced by administration of leukocytes or their products, and in no animal in which leukopenia was induced with sodium citrate solution, argues against the subsequent leukocytosis being a response of the bone marrow stimulated in some manner by the leukopenia. The possibility of the existence of differences in the reaction of the recipient is indicated by the production of leukocytosis in one rabbit but not in another with the same cell suspension.

The chemical nature of the active substances present in solution of lysed leukocytes has not as yet been determined. Preliminary experiments indicate that removal of lipids, including thromboplastin, by ether extraction does not reduce its activity. Considerable loss of activity results from exposure to heating at 66°C for 1 hour.

Summary and conclusions. 1. Leukocytes were obtained in large numbers by introduction of large amounts of physiologic salt solution into the peritoneal cavity of rabbits.

2. Intravenous administration of these cells to the same or different animals was followed by very rapidly developing and severe leukopenia. A subsequent leukocytosis developed in the majority of rabbits after several hours.

3. Disintegrated leukocytes, or aqueous extract of disintegrated leukocytes produced similar results.

4. Preliminary data indicate that the leukocytes stick in the capillaries of the lung. Ether extraction of disintegrated leukocytes does not cause loss of activity but heating reduces activity.

5. These experiments indicate that unsatisfactory preservation of leukocytes in stored blood is not the reason for failure to raise leukocyte counts with transfusions, but that white blood cells contain a substance (or substances) which is capable of producing leukopenia.

We are indebted to Dr. William Holden for the assay of the thromboplastic content of leukocytes.

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Effect of Adrenocorticotrophic Hormone Upon Liver Fat and Urinary Phosphorus in Normal Force-Fed Rat.

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It was shown by Baker, Ingle, Li, and Evans[1] that the administration of pure adrenocorticotrophic hormone (ACTH) to force-fed male rats caused fatty infiltration of the liver as was demonstrated histologically by Sudan stains. This observation was confirmed by chemical analysis of the liver in the present study. In addition, during the administration of ACTH there was noted a significant increase in the urinary excretion of inorganic phosphorus which accompanied a rise in urinary nitrogen and that there was excretion of glucose and loss in body weight. Changes in the weights of certain organs was noted.

Methods. Male rats of the Sprague-Dawley strain were maintained on a diet of Archer Dog Pellets until they reached a weight of approximately 300 g. They were then placed in metabolism cages and maintained on a fluid diet administered by stomach tube each morning (8:30 to 9:15 A.M.) and afternoon (4:15 to 5:00 P.M.). The technic of force-feeding and the diets used were modi-

[1] Baker, B. L., Ingle, D. J., Li, C. H., and Evans, H. M., *Am. J. Anat.*, 1948, **82**, 75.

TABLE I.
Medium Carbohydrate Diet.

Constituent	g
Cellu flour (Chicago Dietetic Supply)	120
Osborne & Mendel salt mixture	40
Diet yeast (Pabst)	100
Wheat germ oil	10
Cod liver oil	10
Vit. K (2-methyl-1,4-naphthoquinone	100 mg
Mazola oil	200
Casein (Labco)	160
Starch	200
Dextrin	190
Sucrose	200
Water to make total of	2000 cc

fications of those described by Reinecke, Ball, and Samuels.[2] The diet was made according to Table I. During the period of adaptation to force-feeding the amount of diet was increased gradually to prevent the development of "food-shock." The animals were brought to a full feeding of 26 cc per day on the 5th day.

The animals were housed in an air-conditioned room in which the temperature was maintained at 74 to 78°F and the humidity at 30 to 35% of saturation. Twenty-four-hour samples of urine were collected at the same hour each day and were preserved with thymol and 1 g of citric acid per sample to insure the acidity of the urines for nitrogen analysis. The following methods of analysis were used: urinary inorganic phosphorus, Müller;[3] urinary non-protein nitrogen by the micro-Kjeldahl procedure; tissue fat and protein, Li, Simpson, and Evans.[4]

The ACTH was prepared by the method of Li, Simpson, and Evans.[5] Following control periods of 14 days, 6 experimental animals were each given 3 mg of ACTH in 7 divided injections every 2 hours during the day for a period of 10 days. One animal was killed by a feeding accident so that the final averages are based upon 5 animals. Six control animals were given injections of physiological saline. At the end of the experiment the animals were anesthetized with ether and exsanguinated. The liver was subjected to lyophilization at low temperature and was then analyzed for fat and protein. Weights were obtained on a number of organs.

Results. The data on body weight, urinary non-protein nitrogen, inorganic phosphorus and glucose are in Fig. 1, the data on organ weights in Table II, and the data on liver composition in Table III. The administration of ACTH caused a significant increase in liver fat so that all of the individual values were higher than any of the control values. The water content of the ACTH livers was correspondingly decreased. The difference between the average protein content of ACTH and control livers was too small to be considered significant.

The injections of ACTH caused a loss of weight associated with a rise in urinary nitrogen, inorganic phosphorus and glycosuria. ACTH caused an increase in the weight of the liver, hypertrophy of the adrenal glands and atrophy of the thymus. The average weights of the organs of the gastrointestinal tract and of the testes were somewhat less in the ACTH series but the kidneys and hearts were heavier. Since the numbers of animals were small the group differences cannot be

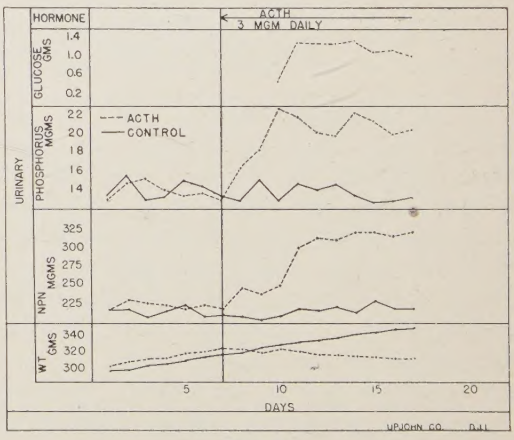

FIG. 1.
The effect of adrenocorticotrophic hormone upon body weight and some constituents of urine in the force-fed normal rat. Averages.

[2] Reinecke, R. M., Ball, H. A., and Samuels, L. T., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 44.

[3] Müller, E., *Hoppe-Seyler's "Zeitsch. fur. Physiol. Chemie,"* 1935, **237**, 35.

[4] Li, C. H., Simpson, M. E., and Evans, H. M., *Growth*, 1948, **12**, 39.

[5] Li, C. H., Simpson, M. E., and Evans, H. M., *J. Biol. Chem.*, 1942, **146**, 627.

TABLE II.
Organ Weight. Grams.

Organ	Adrenocorticotrophic hormone		Controls	
	Avg	Range	Avg	Range
Liver	12.17	10.11-16.21	10.27	9.50-10.80
Stomach	1.33	1.30- 1.40	1.47	1.35- 1.61
Small intestine	5.51	5.11- 5.95	6.04	5.90- 6.40
Cecum and colon	1.74	1.65- 1.80	1.94	1.70- 2.20
Kidneys	2.27	2.10- 2.49	2.01	1.86- 2.01
Testes	3.14	2.65- 3.35	3.32	3.15- 3.45
Heart	1.03	0.97- 1.05	0.94	0.93- 0.97

TABLE III.
Liver Composition of Rats Treated with Adrenocorticotrophic Hormone. Averages and standard deviations of averages.

	Body wt, g	Liver wt, g	Composition in g per 100 g wet liver		
			Water	Fat	Protein
ACTH	315	11.97	61.1	11.96	20.4
	±5.7	±1.1	±1.2	±1.4	±0.6
Control	347	10.27	67.5	5.83	21.73
	±3.1	±0.4	±0.4	±0.6	±0.6

regarded as highly reliable but there was no overlapping in individual values of the two groups in the case of the heart and kidneys and it is probable that ACTH caused these organs to gain weight.

Discussion. There are a number of experimental conditions which cause fatty livers in non-adrenalectomized animals. This response has not been described in the adrenalectomized animal,[6] although Chaikoff *et al.*[7] have reported on the production of fatty livers in hypophysectomized - thyroidectomized dogs. Hartman *et al.*[8] have prepared a fraction of adrenal cortex extracts which sustains the ability of the adrenalectomized rat to deposit fat in the liver. The relationship of adrenal cortical function to the accumulation of liver fat and to other aspects of fat metabolism is not well understood.

One of the objectives of this experiment was to study carcass fat in animals treated with ACTH without the development of glycosuria. It was not anticipated that these animals fed a medium carbohydrate diet would develop glycosuria when given ACTH. The administration of ACTH or of certain adrenal extracts and steroids either inhibits the protein anabolism or stimulates protein catabolism. If the energy represented by the missing protein is not wasted by incomplete metabolism, it must either be stored as fat (the amount of energy which can be stored as extra carbohydrate is very limited) or dissipated by a higher energy output. The effect of ACTH and of cortical hormone overdosage on energy output has not been fully studied. There are two reports[9,10] that 11-dehydrocorticosterone can cause an increase in carcass fat in the mouse. In the present study it was calculated from the extent of rise in urinary nitrogen that the administration of ACTH inhibited the accumulation of approximately 4 g of protein per rat during the 10-day period. During the

6 Ingle, D. J., *J. Clin. Endocrinol.*, 1943, **3**, 603.

7 Chaikoff, I. L., Entenman, C., Rinehart, J. F., and Reichert, F. L., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 170.

8 Hartman, F. A., Brownell, K. A., and Thatcher, J. S., *Endocrinology*, 1947, **40**, 450.

9 Kendall, E. C., Josiah Macy, Jr. Foundation, Conference on Metabolic Aspects of Convalescence, Transactions of 10th Meeting, 1945, 81.

10 Kochakian, C. D., Josiah Macy, Jr., Foundation, Conference on Metabolic Aspects of Convalescence, Transactions of 6th Meeting, 1944, 13.

same period each rat excreted an average of more than 9 g of glucose. Under these conditions an accumulation of carcass fat cannot be expected and this aspect of the problem remains for further study.

The effect of ACTH in causing an increased loss of urinary phosphorus is similar to the effect of 11-dehydro-17-hydroxycorticosterone noted by Ingle and Thorn.[11]

Summary. Normal male rats were force-fed a medium carbohydrate diet. The administration of adrenocorticotrophic hormone in amounts of 3 mg per rat per day for 10 days caused an increase in liver fat, glycosuria, a rise in urinary nitrogen and inorganic phosphorus, suppression of weight gains and some changes in organ weights.

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[11] Ingle, D. J., and Thorn, G. W., *Am. J. Physiol.*, 1941, **132**, 670.